993 ① spectrophotometer ② calibration ③ albumin ④ nitrogen ⑤ aminobenzoic acid ⑥ amylase ⑦ cholesterol ⑧ creatinine ⑨ dextrose ⑩ fibrinogen ⑪ globulin ⑫ haemoglobins ⑬ iodine ⑭ Iron ⑮ oxygen ⑯ phenols ~~⑰~~

Manual of Standardized Procedures for Spectrophotometric Chemistry

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⑰ phosphatase
⑱ phosphorus
⑲ proteins
⑳ sulfanilamide
㉑ sulfate ㉒ uric acid
㉓ body fluid analysis

Manual of Standardized Procedures for Spectrophotometric Chemistry

by **HAROLD J. FISTER**

Director:
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Consulting Chemist:
Reagent Chemicals, Inc., and
Standard Scientific Supply Corp.

with a foreword by
MARGARET A. BAKER, M.D.

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FOREWORD

To one who, for over thirty years, has watched the changing methods and increased scope of clinical chemistry, and can remember when half a dozen of what are now almost routine tests required a full day's work, it is very gratifying to see this excellent compilation of so many valuable procedures. Not only are several methods given for most of the analyses, but the author has personally worked with all the technics until he was satisfied that each one met his own rigid requirements of accuracy. He has adapted them to the several types of photoelectric colorimeters and has given procedures suitable for use with each instrument. Such a comprehensive volume has not previously been available. Many of the methods have been recently developed and are not, as yet, included in any text. The separate pages for notes will be very convenient for personal observations and modifications.

Photoelectric colorimetry removes from readings most of the uncertain factor known as the personal equation. To those who believe that photoelectric instruments are unreliable as well as to those who believe that, because they are instruments of precision, the information obtained by their use is necessarily correct, I would observe that no instrument can take the place of careful and conscientious work, adequate chemical training and good judgment on the part of the technical worker. As the author has commented, the more sensitive the instrument the more glaring the effects of careless technique.

I am very happy for this opportunity to express my appreciation of the author's cooperation and advice during the years I have known him. His extensive and critical reading and his long experience in chemical research and its medical applications have made his assistance most helpful. I feel certain that there is no laboratory worker who will not find in this manual much valuable technical information infrequently, if ever, found in standard texts.

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CONTENTS

The methods in this manual are listed alphabetically and numbered progressively but not necessarily consecutively, the missing numbers being alloted to other procedures scheduled for a future volume.

Method No.	*Determination*	*Calibration Curve*
	ALBUMIN (Biuret Method)	
A-10	Plasma or Serum	A-10 or P-50
	ALBUMIN (Kjeldahl-Nessler Method)	
A-11	Plasma or Serum	A-11 or P-51
	ALBUMIN (Tyrosine Method)	
A-12	Plasma or Serum	A-12 or P-52
A-13	ALBUMIN/GLOBULIN RATIO	Chart A-13
	AMINO ACID NITROGEN, TOTAL (Copper Method)	
A-15a	Whole Blood, Plasma or Serum	A-15
A-15b	Urine	A-15
	AMINO ACID NITROGEN, TOTAL (Naphthoquinone Method)	
A-16	Whole Blood, Plasma or Serum	A-16
	p-AMINOBENZOIC ACID, FREE (Naphthylethylenediamine Method)	
A-20a	Whole Blood	A-20
A-20b	Urine	A-20
	p-AMINOBENZOIC ACID, TOTAL (Naphthylethylenediamine Method)	
A-21a	Whole Blood	A-20
A-21b	Urine	A-20
	p-AMINOBENZOIC ACID, FREE (Thiamine Method)	
A-22	Urine	A-22
	p-AMINOBENZOIC ACID, TOTAL (Thiamine Method)	
A-23	Urine	A-22
A-24	p-AMINOBENZOIC ACID, CONJUGATED	By Calculation
	p-AMINOHIPPURIC ACID (Naphthylethylenediamine Method)	
A-25a	Plasma or Serum	A-25
A-25b	Urine	A-25
	p-AMINOSALICYLIC ACID (Diazo Method)	
A-28a	Whole Blood	A-28
A-28b	Urine	A-28
	AMMONIA NITROGEN (Conway-Phenol-Hypochlorite Method)	
A-30a	Whole Blood	A-30A
A-30b	Urine	A-30B
	AMYLASE (b-Amylose Cotton Method)	
A-35	Plasma or Serum	A-35
	AMYLASE (Iodine-Starch Method)	
A-36a	Plasma or Serum	By Calculation
A-36b	Urine	By Calculation
	AMYLASE (Starch Substrate Method)	
A-37a	Plasma or Serum	A-37
A-37b	Whole Blood	A-37

Method No.	*Determination*	*Calibration Curve*
	ASCORBIC ACID (Dichlorobenzenone Indophenol Method)	
A-45a	Plasma or Serum	A-45
A-45b	Urine	A-45
	ASCORBIC ACID (Dinitrophenylhydrazine Method)	
A-46a	Whole Blood, Plasma or Serum	A-46
A-46b	Urine	A-46
	BARBITURIC ACID (Cobalt Method)	
B-5	Urine	B-5
	BILIRUBIN (Diazo Method) TOTAL TOTAL DIRECT PROMPT DIRECT DELAYED DIRECT INDIRECT PERCENTAGE PROMPTNESS DIRECT REACTION	
B-10	Plasma or Serum	B-10
	BILIRUBIN (Diazo Method)	
B-11	Urine	B-11
	BISMUTH (Iodide Method)	
B-15	Urine	B-15
	BROMIDE (Gold Chloride Method)	
B-20a	Serum	B-20
B-20b	Urine	B-20
B-20c	Cerebrospinal Fluid	B-20
	BROMSULPHALEIN (Greene and Rosenthal Technics)	
B-25	Plasma or Serum	B-25
	CALCIUM (Phosphate Method)	
C-10a	Plasma or Serum	C-10 or P-36
C-10b	Urine	C-10 or P-36
C-10c	Feces	C-10 or P-36
	CARBON DIOXIDE CAPACITY (Diphenylcarbazide Method)	
C-15	Plasma	C-15
	CARBON DIOXIDE CONTENT (Diphenylcarbazide Method)	
C-16	Plasma	C-15
	CARBON MONOXIDE (Palladium Method)	
C-20	Whole Blood	C-20
	CHLORIDE (Silver Iodate Method)	
C-25a	Plasma or Serum	C-25 and C-25A
C-25b	Urine	C-25
C-25c	Cerebrospinal Fluid	C-25
C-25d	Gastric Contents	C-25
	CHOLESTEROL, TOTAL (Acetic Anhydride Method)	
C-30	Plasma or Serum	C-30
	CHOLESTEROL, ESTER (Acetic Anhydride Method)	
C-31a	Plasma or Serum	C-31
	CHOLESTEROL, FREE (Acetic Anhydride Method)	
C-31b	Plasma or Serum	By Calculation

Method No.	Determination	Calibration Curve
	CHOLESTEROL, TOTAL (Acetyl Chloride Method)	
C-32	Plasma or Serum	C-32
	CHOLESTEROL, FREE (Acetyl Chloride Method)	
C-33a	Plasma or Serum	C-32
	CHOLESTEROL, ESTER (Acetyl Chloride Method)	
C-33b	Plasma or Serum	By Calculation
	COPPER (Diethyldithiocarbamate Method)	
C-35	Whole Blood, Plasma or Serum	C-35
	CREATINE (Dinitrobenzoic Acid Method)	
C-40a	Whole Blood, Plasma or Serum	C-42
C-40b	Urine	C-42
	CREATINE (Alkaline Picrate Method)	
C-41a	Whole Blood, Plasma or Serum	C-43
C-41b	Urine	C-43
	CREATININE (Dinitrobenzoic Acid Method)	
C-42a	Whole Blood, Plasma or Serum	C-42
C-42b	Urine	C-42
	CREATININE (Alkaline Picrate Method)	
C-43a	Whole Blood, Plasma or Serum	C-43
C-43b	Urine	C-43
	"DEXTROSE," TOTAL REDUCING SUBSTANCES (Alkaline Copper-Phosphomolybdate Method)	
	Whole Blood, Plasma or Serum	
D-10a	Macro	D-10
D-10b	Micro	D-10
D-10c	Urine	D-10
D-10d	Cerebrospinal Fluid	D-10
	DEXTROSE, TRUE (Alkaline Copper-Arsenomolybdate Method)	
	Whole Blood, Plasma or Serum	
D-12a	Macro	D-12A
D-12b	Micro	D-12B
	DEXTROSE (Ferricyanide-Ferric Iron Method)	
	Whole Blood, Plasma or Serum	
	TRUE DEXTROSE	
D-14a	Macro	D-14A
D-14b	Micro	D-14B
	TOTAL REDUCING SUBSTANCES	
D-14c	Macro	D-14A
D-14b	Micro	D-14B
	"DEXTROSE" (Sodium Carbonate Method)	
D-16	Urine	D-16
	ETHYL ALCOHOL (Dichromate Method)	
E-10a	Whole Blood	E-10
E-10b	Urine	E-10
	FIBRINOGEN (Biuret Method)	
F-10	Plasma	F-10 or P-50
	FIBRINOGEN (Kjeldahl-Nessler Method)	
F-11	Plasma	F-11 or P-51

Method No.	*Determination*	*Calibration Curve*
	FIBRINOGEN (Tyrosine Method)	
F-12	Plasma	F-12 or P-52
	GALACTOSE (Alkaline Copper-Phosphomolybdate Method)	
G-10a	Whole Blood, Plasma or Serum	G-10 or D-12A
G-10b	Urine	G-10 or D-12A
	GLOBULIN (Biuret Method)	
G-15	Plasma or Serum	G-15 or P-50
	GLOBULIN (Kjeldahl-Nessler Method)	
G-16	Plasma or Serum	G-16 or P-51
	GLOBULIN (Tyrosine Method)	
G-17	Plasma or Serum	G-17 or P-52
	GUANIDINE (Nitroprusside-Ferricyanide Method)	
G-20a	Whole Blood	G-20A
G-20b	Urine	G-20B and Chart G-20
	HEMOGLOBIN (Acid Hematin Method)	
H-10	Whole Blood	H-10
	HEMOGLOBIN (Alkaline Hematin Method)	
H-11	Whole Blood	H-11
	HEMOGLOBIN (Indirect Iron Method)	
H-12	Whole Blood	I-27 or I-28 and Chart H-12
	HEMOGLOBIN (Indirect Oxygen Method)	
H-13	Whole Blood	O-10 and Chart H-13
	HEMOGLOBIN, OXY- (Sodium Carbonate Method)	
H-14	Whole Blood	H-14
	HEMOGLOBIN, TOTAL (Ferricyanide Method)	
H-15	Whole Blood	H-15
H-17	HEMOGLOBIN PERCENTAGE	Chart H-17
	HIPPURIC ACID (Hypobromite Method)	
H-20	Urine	H-20
	INDICAN (Ferric Chloride Method)	
I-10a	Plasma or Serum	I-10
I-10b	Urine	I-10
	INULIN (Diphenylamine Method)	
I-15a	Plasma or Serum	I-15
I-15b	Urine	I-15
	INULIN (Resorcinol-Thiourea Method)	
I-16a	Plasma or Serum	I-16
I-16b	Urine	I-16
	IODINE, TOTAL (Ceric Sulfate-Catalytic Method)	
I-19a	Whole Blood, Plasma or Serum	I-19A or I-19B
I-19b	Urine	I-19A or I-19B
	IODINE, PROTEIN-BOUND (Ceric Sulfate-Catalytic Method)	
I-20	Whole Blood, Plasma or Serum	I-19A or I-19B
	IODINE, INORGANIC (Ceric Sulfate-Catalytic Method)	
I-21	Whole Blood, Plasma or Serum	I-19A or I-19B
	IRON, NON-HEMOGLOBIN (Dipyridyl Method)	
I-25	Plasma or Serum	I-25
	IRON, NON-HEMOGLOBIN (o-Phenanthroline Method)	
I-26	Plasma or Serum	I-26

Method No.	Determination	Calibration Curve
	IRON, TOTAL (Mercaptoacetate Method)	
I-27	Whole Blood	I-27
	IRON, TOTAL (Thiocyanate Method)	
I-28	Whole Blood	I-28
	17-KETOSTEROIDS, TOTAL (Dinitrobenzene Method)	
K-10a	Urine	K-10
	17-KETOSTEROIDS, TOTAL (Girard Reagent-Dinitrobenzene Method)	
K-10b	Urine	K-10
	LACTIC ACID (Hydroquinone Method)	
L-10a	Whole Blood, Plasma or Serum	L-10
L-10b	Urine	L-10
	LACTIC ACID (Hydroxybiphenyl Method)	
L-11	Whole Blood, Plasma or Serum	L-11
	LEAD (Dithizone Method)	
L-15	Urine	L-15A and L-15B
	MAGNESIUM (Titan Yellow Method)	
M-10a	Plasma or Serum	M-10
M-10b	Whole Blood	M-10
M-10c	Urine	M-10
	MERCURY (Dithizone Method)	
M-15	Whole Blood or Urine	M-15
	NICOTINIC ACID (Cyanogen Bromide Method)	
N-10	Erythrocytes	N-10
	NICOTINIC ACID (Cyanogen Bromide Method)	
	Urine	
N-11a	EXCLUSIVE OF TRIGONELLINE	N-11
N-11b	INCLUDING TRIGONELLINE	N-11
	NITROGEN, NONPROTEIN (Kjeldahl-Nessler Method)	
N-15a	Whole Blood, Plasma or Serum	N-15
N-15b	Urine	N-15
	NITROGEN, TOTAL (Kjeldahl-Nessler Method)	
N-16a	Whole Blood	N-16A
N-16b	Plasma or Serum	N-16A or N-16B
N-16c	Urine	N-16A
	OXYGEN CAPACITY (Sulfosalicylic Acid Method)	
O-10	Whole Blood	O-10
	OXYGEN CAPACITY (Indirect Iron Method)	
O-11	Whole Blood	I-27 or I-28
	OXYGEN CONTENT (Sulfosalicylic Acid Method)	
O-12	Whole Blood	O-10
	PHENOL, FREE (Nitroaniline Method)	
P-10a	Whole Blood	P-10
P-10b	Urine	P-10
	PHENOL, CONJUGATED (Nitroaniline Method)	
P-11a	Whole Blood	P-10
P-11b	Urine	P-10
	PHENOL, TOTAL (Nitroaniline Method)	
P-12a	Whole Blood	P-10
P-12b	Urine	P-10

Method No.	*Determination*	*Calibration Curve*
	PHENOLSULFONPHTHALEIN	
	Urine	
P-20a	In the absence of blood and/or bile pigments	P-20
P-20b	In the presence of blood and/or bile pigments	P-20
	PHOSPHATASE, ACID AND ALKALINE (Bodansky Method)	
P-25	Serum	P-25 or P-36
	PHOSPHATASE, ACID (Gutman Method)	
P-27	Serum	P-30
	PHOSPHATASE, ACID AND ALKALINE (King-Armstrong Method)	
P-30	Serum	P-30
	PHOSPHATASE, ACID AND ALKALINE (Shinowara-Jones-Reinhart Method)	
P-32	Serum	P-32 or P-36
	PHOSPHORUS, TOTAL ACID-SOLUBLE (Aminonaphthol Sulfonic Acid Method)	
P-35	Whole Blood, Plasma or Serum	P-35 or P-36
	PHOSPHORUS, INORGANIC (Aminonaphthol Sulfonic Acid Method)	
P-36a	Whole Blood, Plasma or Serum	P-36
P-36b	Urine	P-36
	PHOSPHORUS, LIPOID (Aminonaphthol Sulfonic Acid Method)	
P-37	Whole Blood, Plasma or Serum	P-37 or P-36
	PHOSPHORUS, TOTAL (Aminonaphthol Sulfonic Acid Method)	
P-38a	Whole Blood, Plasma or Serum	P-38 or P-36
P-38b	Urine	P-38 or P-36
	POTASSIUM (Cobaltinitrite Method)	
P-40	Serum	P-40
P-45	POTASSIUM PERMANGANATE—VOLUMETRIC STANDARDIZATION	P-45
	PREGNANDIOL (Acetyl Chloride Method)	
P-47	Urine	P-47
	PREGNANDIOL (Sulfuric Acid Method)	
P-48	Urine	P-48
	PROTEIN, TOTAL (Biuret Method)	
P-50a	Plasma or Serum	P-50A
P-50b	Cerebrospinal Fluid	P-50B
P-51	PROTEIN, TOTAL (Kjeldahl-Nessler Method)	By Calculation
	PROTEIN, TOTAL (Tyrosine Method)	
P-52a	Plasma or Serum	P-52A
P-52b	Cerebrospinal Fluid	P-52B
	PROTEIN, TOTAL (Nephelometric Methods)	
P-53	Cerebrospinal Fluid	P-53
P-54	Urine	P-54
	PYRUVIC ACID (Dinitrophenylhydrazine Method)	
P-60a	Whole Blood	P-60
P-60b	Urine	P-60

Method No.	*Determination*	*Calibration Curve*
	SALICYLIC ACID (Ethylene Dichloride-Ferric Nitrate Method)	
S-10	Plasma or Serum	S-10
	SODIUM (Uranium Zinc Acetate Method)	
S-15	Serum	S-15 and S-15A
	SULFANILAMIDE, FREE (Naphthylethylenediamine Method)	
S-22a	Whole Blood, Plasma or Serum	S-22
S-22b	Urine	S-22
S-22c	Cerebrospinal Fluid	S-22
	SULFANILAMIDE, TOTAL (Naphthylethylenediamine Method)	
S-23a	Whole Blood, Plasma or Serum	S-22
S-23b	Urine	S-22
S-23c	Cerebrospinal Fluid	S-22
S-24	SULFANILAMIDE, ACETYLATED	By Calculation
	SULFACETIMIDE (Naphthylethylenediamine Method)	
S-26a	FREE	S-26 or S-22
S-26b	TOTAL	S-26 or S-22
S-26c	ACETYLATED	By Calculation
	SULFADIAZINE (Naphthylethylenediamine Method)	
S-28a	FREE	S-28 or S-22
S-28b	TOTAL	S-28 or S-22
S-28c	ACETYLATED	By Calculation
	SULFAGUANIDINE (Naphthylethylenediamine Method)	
S-30a	FREE	S-30 or S-22
S-30b	TOTAL	S-30 or S-22
S-30c	ACETYLATED	By Calculation
	SULFAMERAZINE (Naphthylethylenediamine Method)	
S-32a	FREE	S-32 or S-22
S-32b	TOTAL	S-32 or S-22
S-32c	ACETYLATED	By Calculation
	SULFAPYRIDINE (Naphthylethylenediamine Method)	
S-42a	FREE	S-42, S-48 or S-22
S-42b	TOTAL	S-42, S-48 or S-22
S-42c	ACETYLATED	By Calculation
	SULFASUXIDINE (Naphthylethylenediamine Method)	
S-44a	"TOTAL"	S-44 or S-48
S-44b	"COMBINED"	By Calculation
	SULFATHIAZOLE, FREE (Naphthylethylenediamine Method)	
S-48a	Whole Blood, Plasma or Serum	S-48
S-48b	Urine	S-48
S-48c	Cerebrospinal Fluid	S-48
	SULFATHIAZOLE, TOTAL (Naphthylethylenediamine Method)	
S-49a	Whole Blood, Plasma or Serum	S-48
S-49b	Urine	S-48
S-49c	Cerebrospinal Fluid	S-48

Method No.	*Determination*	*Calibration Curve*
S-50	SULFATHIAZOLE, ACETYLATED	By Calculation
	SULFATE, INORGANIC (Naphthylethylenediamine Method)	
S-60a	Whole Blood, Plasma or Serum	S-60
S-60b	Urine	S-60
	SULFATE, TOTAL (Naphthylethylenediamine Method)	
S-61a	Whole Blood, Plasma or Serum	S-60
S-61b	Urine	S-60
S-62	SULFATE, ETHEREAL	By Calculation
	SULFUR, TOTAL (Naphthylethylenediamine Method)	
S-63a	Whole Blood, Plasma or Serum	S-63 or S-60
S-63b	Urine	S-63 or S-60
S-64	SULFUR, NEUTRAL	By Calculation
	THIOCYANATE (Ferric Nitrate Method)	
T-10	Plasma or Serum	T-10
	THIOURACIL (Grote Reagent Method)	
T-15a	Whole Blood, Plasma or Serum	T-15
T-15b	Urine	T-15
	THYMOL TURBIDITY (Nephelometric Method)	
T-20	Serum	T-20
	UREA NITROGEN (Diacetylmonoxime Method)	
U-10a	Whole Blood, Plasma or Serum	U-10
U-10b	Urine	U-10
	UREA NITROGEN (Urease-Nessler Method)	
U-11a	Whole Blood, Plasma or Serum	U-11
U-11b	Urine	U-11
	URIC ACID (Cyanide-Urea Method)	
U-15a	Plasma or Serum	U-15
U-15b	Whole Blood	U-15
U-15c	Urine	U-15
	URIC ACID (Glycerol-Silicate Method)	
U-16a	Plasma or Serum	U-16
U-16b	Urine	U-16
	UROBILINOGEN (Aldehyde Method)	
	Urine	
U-20a	In the absence of bilirubin and/or protein	U-20
U-20b	In the presence of bilirubin and/or protein	U-20
	Feces	
U-20c	Simplified procedure	U-20
U-20d	Ether extraction procedure	U-20
	VOLUME, TOTAL BLOOD (Evans Blue Method)	
V-50	Plasma	By Calculation
	ZINC TURBIDITY (Nephelometric Method)	
Z-10	Serum	T-20
	APPENDIX	
	Optical Density-Transmission Table	Chart X-1
	Cuvette Diameters	
	Preparation of Miscellaneous Solutions	
	Table of Normal Values.	

Manual of Standardized Procedures for Spectrophotometric Chemistry

INTRODUCTION

In modern analytical chemistry, the spectrophotometer is replacing the photoelectric colorimeter, just as the latter replaced the visual colorimeter which, in turn, outmoded the comparator block. In addition to several excellent books that cover the theory of spectral analysis, the manufacturers of spectrophotometers issue booklets supplying the technical information necessary to operate these instruments, but no comprehensive collection of analytical procedures adapted for spectrophotometry has as yet appeared. Because of a constantly increasing number of requests for a wide variety of technical procedures and calibration curves, the author has endeavored to collate under one cover a selection of methods adequate for the needs of the clinical laboratory and to present them in such a manner that they may be readily followed by those with a minimum of experience in the use of the spectrophotometer. As may be seen from the bibliography, the pertinent original articles are widely scattered throughout the literature; in two instances, translations of the original have not yet appeared in English.

The selection of a group of methods for a manual of this type presented some difficulties; no single method satisfies all conditions without modification. With certain methods, because of their simplicity, widespread use or sound chemical basis, the choice was obvious; others were chosen only after due consideration of the intended purpose. For many substances more than one analytical procedure has been outlined for reasons of preference, economy or accuracy. Many workers, who have definite preferences for certain methods with which they are thoroughly familiar and with which they obtain excellent results, hesitate to adopt new procedures. In some laboratories, costly and elaborate set-ups exist for the carrying out of various determinations, thus rendering the adoption of a new procedure uneconomical. The deciding factor when choosing a method is the technical skill required of the analyst; in some instances a simple and rapid procedure is outlined for routine work and a more exacting technic for precise work. The type of sample to be analyzed as well as the conditions under which the analysis must be made governs, to a large extent, the method of analysis to be followed. A choice of methods is advantageous in that it enables one to check the results obtained by one procedure with those by another in which the analytical principles differ. No attempt has been made to select only those methods capable of yielding results of maximum accuracy; the manual is intended for routine work and as an introduction to the field of spectrophotometric analysis and not for specialists in the various fields of research work.

Each method is treated in a similar manner. The analytical principles are discussed very briefly; since this is a working manual and not a text, theoretical considerations are held to a minimum. The type of sample, its collection and the method of preservation are outlined; substances which cause interference are noted. Any special apparatus required is listed and precautions necessary in cleaning or handling described. The preparation of all reagents is given in detail with notes as to preservation and stability. It will be noted that whenever a chemical is mentioned, the formula, grade and source of supply immediately follow. The methods and curves in this manual were prepared with these materials. This is not to be taken as an indication that these chemicals, and these only, are satisfactory; materials obtained from other manufacturers may be used in many instances but in some specific cases the author has found none other than the material specified to be suitable. Many reagents ordinarily prepared as percentage solutions have been placed on an accurate normal or molar basis. The test procedure is outlined in a step by step form with the special details of technic and the precautions to be observed clearly noted. Indefinite intervals of time and measurements of volume have been eliminated; where calculations are necessary, the formulae are given. Some steps and precautions have been included which may seem not only superfluous, but even slightly ridiculous, to the experienced worker. However, it should be remembered that this manual is designed and outlined for the use of those with little or no experience with spectrophotometric technic. Considering the comparatively short time in which spectrophotometers have been used for other than research work, the majority of routine analyses are at present made by workers with limited experience in this field.

The calibration procedure, including the preparation of the required standards, follows the test procedure, every step in both being so carefully controlled that the original calibration curve may be closely approximated, if not exactly reproduced. It cannot be too strongly emphasized that in reproducing a calibration curve every detail of technic must be identical with that employed in the preparation of the original curve. *The author wishes to point out that the curves in the manual are not offered as a calibration for any instrument other than his own, but are intended as a guide to the analyst when preparing his individual curves.* The author is in accord with the statement of Mellon that "An experienced worker would not use the curve of another instrument without checking" and that of Sandell who states that "only the most sanguine user of a spectrophotometer will calculate the concentration of his colored solution from the observed extinction and the value of the extinction taken from the literature."

However, the curves appearing in the manual may be used with Coleman spectrophotometers and the error will fall well within the limits permitted for routine clinical work, **if the conditions enumerated below are carefully observed:**

1. The wave band must be so accurately adjusted that a reading at the specified wave length agrees within ±0.5% of that stamped on the calibrating filter furnished by the manufacturer. In all procedures the wave-length setting and stray light filter must be as specified.

2. Cuvettes must be of the same diameter and from the same series as those employed in the original calibration.
3. The reagents must be prepared exactly as outlined and from the same materials as listed, until the use of chemicals other than those specified has been found to be satisfactory. Precautions regarding stability and preservation must be observed.
4. The volumetric glassware, as is repeatedly stated throughout the manual, must be within the tolerances specified by the National Bureau of Standards. In the author's laboratory only "Normax Precision" volumetric glassware is used, each single piece of which is checked upon receipt against a master set of glassware certified by the National Bureau of Standards. Next to the training and experience of the worker and the use of accurately prepared reagents, the most important factor in attaining a high degree of accuracy and reproducible results is the use of accurately calibrated glassware. Where special pipettes are called for (as for the measurement of chloroform and for rapid delivery), these must be used. Mohr measuring pipettes, especially the 1 and 2 ml sizes, are preferable to transfer pipettes when preparing calibration standards.
5. All technical details must be identical with those described. The inability to reproduce a curve will generally be found to be due to a deviation in individual technic, such as the handling and drainage of a pipette, the reading of a meniscus, disregard for accurate time or temperature measurements or as a result of "short-cuts."

Even though the technical details of any procedure are under such perfect control that reliance can be placed upon a calibration curve which has been repeatedly reproduced, a standard or series of standards should be run at regular intervals as a performance check. For some procedures standards *must* be run simultaneously with the sample and these are so noted.

Although entire calibration curves from zero concentration to a variable maximum have usually been plotted to illustrate the slope of the curve, only that portion of any curve, whether a straight line or not, falling between 10 and 80% transmission should be used and for maximum accuracy, only that portion between 20 and 60%. In a few instances where the normal concentration range of a certain substance is very narrow and far less than the possible pathological increase, the transmission readings for normal values may fall between 80 and 100% transmission and due cognizance must be taken of the fact that there is a large inherent instrumental error when measurements are made on this portion of the scale. The wave length selected is, as a rule, the wave length of maximum absorption of the colored solution which is to be measured. In certain instances, where a range of application is more important than maximum accuracy and in order to include the normal range with sufficient margin on either side to cover the usual pathological variations, a wave length is used which is not at the peak of maximum absorption but on the slope of the curve. The same expedient is used to reduce blank values or eliminate the effect of interfering substances. In the majority of the curves included in the manual the entire curve, as plotted, or that portion of the curve of particular interest will be found to be a straight line and obey the Bouguer-Beer law. While linearity is advantageous, particularly in two component systems and where a K value is used to calculate the concentration, it is not essential for maximum accuracy. In the preparation of some of the original calibration curves, preference was given to a suitable concentration range rather than to linearity. The author prefers to read concentrations from a calibration table rather than calculate by means of a K value. Once a table has been carefully prepared from an accurately constructed curve, the chance of error due to miscalculation is minimized.

For the many thousands of laboratories which do not as yet own a spectrophotometer but use photoelectric colorimeters, adaptation procedures for both linear scale and logarithmic scale instruments have been appended to each method. The character of the curve obtained with a photoelectric colorimeter will not always be the same as that illustrated, due to the width of the absorption band of the filter used.

It may seem that there is an undue amount of repetition, especially in the description of the preparation of certain reagents and in occasional technical procedures which appear in more than one method. Since the methods have been available singly and in special sets, each method must necessarily be complete in itself. This manner of presentation also allows for the insertion of new methods and the deletion of older ones without necessitating extensive revision.

The author wishes to express his appreciation for the interest, advice and assistance of all who have aided in the preparation of the manual. He has endeavored to acknowledge the source of all pertinent data and regrets any oversight that may have occurred. He is particularly indebted to Dr. Margaret A. Baker who was so kind as to write the foreword; to Miss Ida T. Belmonte for her painstaking work in performing many hundreds of lead analyses; to Miss Adele Newfield who was responsible for preparing and checking the manuscript and assisted in proofreading; to Mr. J. Baldwin MacNemar without whose assistance and encouragement the manual would not have reached completion; and to the publishers, Standard Scientific Supply Corporation, for their cooperation in placing the manual in the hands of those who, the author hopes, may find it of some value.

Brooklyn, New York
January 1950

H. J. F.

ALBUMIN

A spectrophotometric adaptation based on the methods and modifications of Kingsley (1939; 1940; 1942), Maclay and Osterberg (1940), Robinson and Hogden (1940), Weichselbaum (1946) and Gornall, Bardawill and David (1949)

PRINCIPLE

Albumin is separated from globulin by precipitation of the latter with sodium sulfate, ether being used to reduce the density of the precipitated globulin thus permitting the final separation to be made by centrifugation. The albumin in the globulin-free centrifugate is determined by treatment with copper sulfate in the presence of alkaline sodium potassium tartrate to form a reddish-purple complex (biuret reaction).

DETERMINATION IN PLASMA OR SERUM

SAMPLE

Plasma or serum from blood withdrawn during post-absorptive state. Tourniquet must be removed immediately after entering vein and collection of blood should be completed within one minute. Anticoagulant—potassium oxalate. There must be no trace of hemolysis. Plasma or serum must be separated from the red blood cells as soon as possible.

TEST PROCEDURE

Special Apparatus

Glass-stoppered centrifuge tubes, 15 ml.
Ostwald-Folin pipettes accurately calibrated "to contain" 0.5 ml.
Capillary pipettes, Wintrobe type.
Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.

Reagents

1. **Sodium Sulfate, 23% Aqueous.** Heat approximately 200 ml distilled water to 37° C.; half fill a 100 ml volumetric flask with the warm water; add 23 gm anhydrous sodium sulfate (Na_2SO_4—Baker & Adamson, Reagent Special); add more warm water until the body of the flask is just about filled; stopper and shake until solution is complete; dilute to volume with the warm water. Keep in incubator at 37° C.

Note: Reagent grade sodium sulfate may vary considerably in its free acid or free alkali content. Each new lot should be tested as follows: dissolve 5 gm in 60 ml carbon dioxide-free distilled water and add 3 drops phenolphthalein indicator solution—no pink color should appear; add 0.5 ml 0.02 N sodium hydroxide—a pink color should appear. Sodium sulfate which does not show these characteristics is unsatisfactory for the differential precipitation of proteins and should be discarded—the reaction of the final solution must be just on the acid side of neutrality.

2. **Ether, Anhydrous** ($(C_2H_5)_2O$—Mallinckrodt, A.C.S.).
3. **Biuret Reagent (Gornall-Bardawill-David).** In a 1000 ml volumetric flask place 1.5 gm crystalline copper sulfate ($CuSO_4 \cdot 5H_2O$—Baker & Adamson, A.C.S., Fine Crystals) and 6 gm sodium potassium tartrate ($NaKC_4H_4O_6 \cdot 4H_2O$—Baker & Adamson, A.C.S.); add approximately 500 ml distilled water and shake until dissolved; add with constant agitation of the flask, 300 ml 2.5 N sodium hydroxide (a) and mix; add 1 gm potassium iodide (KI—Mallinckrodt, A.C.S.) and shake until dissolved; dilute to volume with distilled water. Preserve in ceresin-lined bottle. Stability of a properly prepared reagent is indefinite—discard if a black or reddish precipitate forms.
   (a) Sodium hydroxide, volumetric 2.5 N; prepare by diluting slightly more than the required amount of concentrated carbonate-free sodium hydroxide solution (see Appendix); dilute 20 ml of this approximately 2.5 N solution to 100 ml; titrate against standard 0.5 N acid using phenolphthalein as indicator; adjust accordingly and re-titrate —20 ml of 0.5 N acid require 20 ml of a 1:5 dilution of 2.5 N alkali. Preserve in ceresin-lined bottle.

Technic

Note: If globulin is to be estimated by actual determination (Method G-15) instead of by calculation, reserve the precipitate in Step 7.

1. Place a 15 ml glass-stoppered centrifuge tube in a 150 ml beaker half-filled with warm water at 37° C.
2. Without removing the tube from the beaker, add 7.5 ml 23% sodium sulfate (1), removed from the incubator immediately before use.
3. Add 0.5 ml sample from a warm Ostwald-Folin pipette, thoroughly rinsing out the pipette by drawing in and expelling some of the mixture several times.
4. Stopper the tube and mix the contents by inversion.
5. Add 4 ml ether (2), stopper the tube and shake vigorously for 30 seconds.
6. Centrifuge the unstoppered tube at 2000 rpm for 15 minutes—the precipitated globulin is tightly packed in a layer at the interface between the sulfate solution and the ether.

7. Slant the tube so that the globulin layer is separated from the walls; remove slightly more than 2 ml of the sulfate layer—this is best done by inserting a Wintrobe-type capillary pipette through the ether layer along the lower wall of the tube into the sulfate layer, holding the index finger over the top of the pipette to prevent the ingress of any ether—RESERVE PRECIPITATE FOR GLOBULIN ANALYSIS (Method G-15).
8. In an 18 × 150 mm Pyrex test tube (sample) place
   2 ml of the sulfate layer from Step 7, carefully wiping the pipette clean of any adherent protein particles.
9. In another 18 × 150 mm Pyrex test tube (reagent blank) place
   2 ml 23% sodium sulfate (1).
10. To each tube add 8 ml biuret reagent (3) and mix by whirling.
11. Place tubes in water bath at 25° C. for 30 minutes.

Note: An occasional highly lipemic sample may show a turbidity at this point; add an equal volume of petroleum ether to the colored solution and extract the lipids by gently inverting the tube until the aqueous phase is clear; centrifuge at 1500 rpm for 3 minutes, transfer the aqueous layer to a cuvette and proceed with Step 12.

12. Measure the transmittancy of the sample against the reagent blank set at 100% transmission at a wave length of **540** mμ and multiply the value from the calibration table for total protein (Method P-50) by 0.8 to obtain the concentration of albumin in per cent. If a calibration table has been prepared for albumin, the concentration is obtained directly.

CALIBRATION PROCEDURE

Note: The following calibration procedure is optional. The values in the calibration table for total protein (Method P-50), when multiplied by 0.8 are equivalent to the concentration of albumin in per cent.

Special Apparatus

Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.

Reagents

3. **Biuret Reagent (Gornall-Bardawill-David).** Same as for Test Procedure.
4. **Sodium Chloride, 0.85% Aqueous.** Dissolve 8.5 gm sodium chloride (NaCl—Merck, Special for Biological Work) in distilled water and dilute to 1000 ml.
5. **Albumin Standards**
   (a) Protein stock standard. Determine the total protein in a well mixed sample of pooled human sera by the Kjeldahl procedure (Method P-51).
   (b) Calibration standards. In a series of four accurately calibrated 100 ml volumetric flasks place respectively, volumes of pooled sera (5a) containing 120, 240, 360 and 480 mg protein; dilute to volume with 0.85% sodium chloride (4). These standards represent concentrations of albumin equivalent to 1.92, 3.84, 5.76 and 7.68%. Prepare just before use.

Technic

1. Transfer 2 ml of each calibration standard (5b) to a correspondingly marked 18 × 150 mm Pyrex test tube.
2. In a fifth 18 × 150 mm Pyrex test tube (zero concentration standard) place 2 ml 0.85% sodium chloride (4).
3. Proceed exactly as outlined in the Test Procedure, Steps 10–11 inclusive.
4. Remove tubes from water bath and immediately set the zero concentration standard at 100% transmission at a wave length of **540** mμ and record the transmittancy of the 1.92, 3.84, 5.76 and 7.68% standards. The transmittancy of the 7.68% standard should be approximately 45.5%.
5. Plot the observed values on semi-log graph paper —the points fall on a straight line which passes through the origin (Calibration Curve A-10).
6. Prepare a calibration table from the T-C values obtained from the plotted curve.

◆

PROCEDURE FOR USE WITH LINEAR-SCALE PHOTOELECTRIC COLORIMETERS (SHEARD-SANFORD TYPE)

TEST PROCEDURE

Exactly as outlined; set the reagent blank at 100 and use a green filter.

CALIBRATION PROCEDURE

Exactly as outlined—use a green filter; plot the observed readings on semi-log graph paper—the points fall on a straight line passing through the origin.

◆

PROCEDURE FOR USE WITH LOGARITHMIC-SCALE PHOTOELECTRIC COLORIMETERS (KLETT-SUMMERSON TYPE)

TEST PROCEDURE

Exactly as outlined; set the reagent blank at 0 and use a green filter.

CALIBRATION PROCEDURE

Exactly as outlined—use a green filter; plot the observed readings on linear graph paper—the points fall on a straight line passing through the origin.

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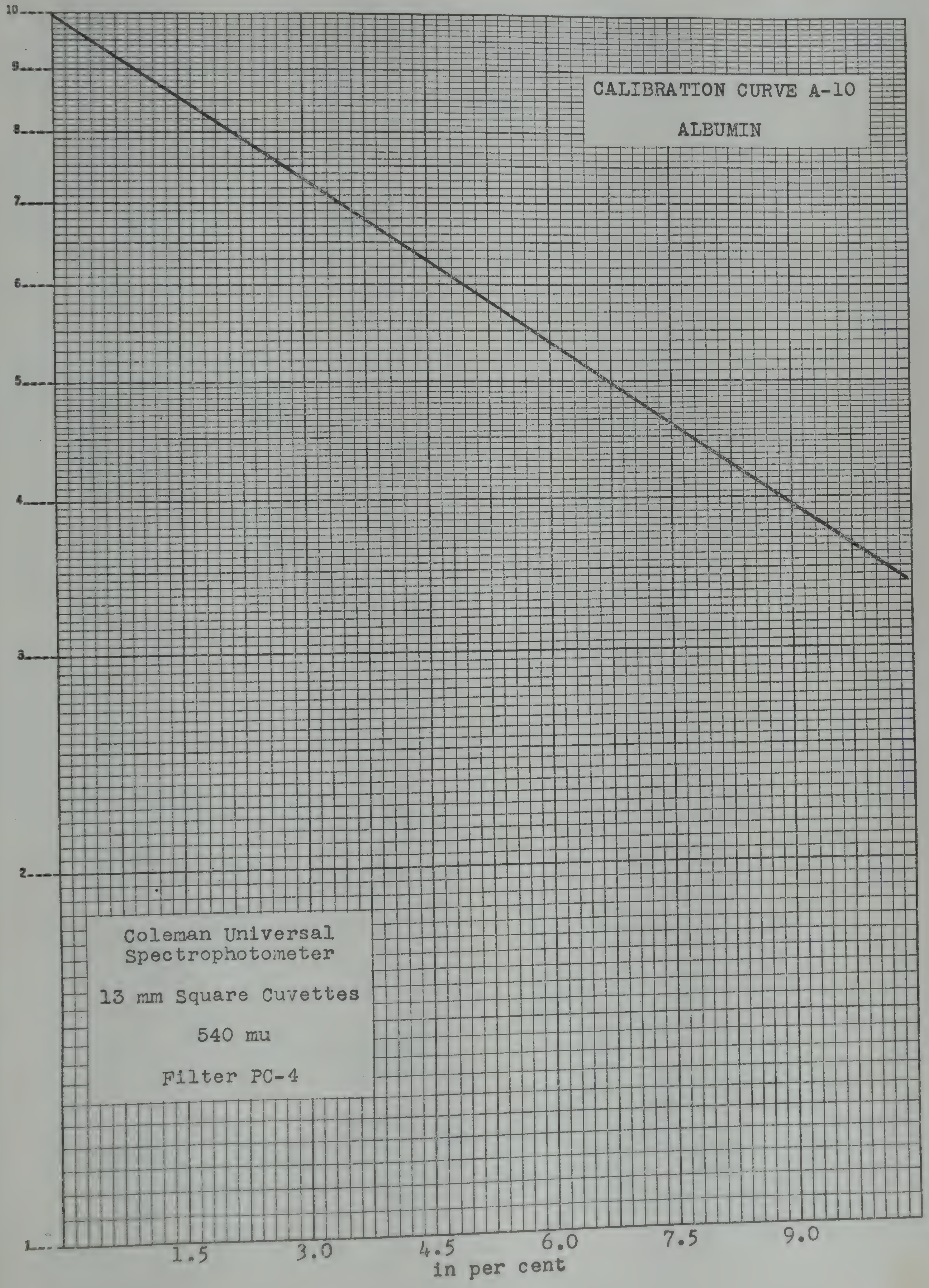
CALIBRATION CURVE A-10
ALBUMIN
Coleman Universal Spectrophotometer
13 mm Square Cuvettes
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Filter PC-4
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Meter Reading	0	1	2	3	4	5	6	7	8	9
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ALBUMIN

A spectrophotometric adaptation based on the methods and modifications of Folin and Wu (1919), Howe (1921), Myers (1921), Campbell and Hanna (1937), Hoffman and Osgood (1940), Kingsley (1940) and Maclay and Osterberg (1940)

PRINCIPLE

Albumin is separated from globulin by precipitation of the latter with sodium sulfate, ether being used to reduce the density of the precipitated globulin thus permitting the final separation to be made by centrifugation. The albumin in the globulin-free centrifugate is digested with a selenium-sulfuric acid mixture with the aid of hydrogen peroxide to convert nitrogenous constituents to ammonium sulfate in which the ammonia is determined by means of the reddish-orange color developed with alkaline potassium mercuric iodide (Nessler reaction).

DETERMINATION IN PLASMA OR SERUM

SAMPLE

Plasma or serum from blood withdrawn during postabsorptive state. Tourniquet must be removed immediately after entering vein and collection of blood should be completed within one minute. Anticoagulant—potassium oxalate. There must be no trace of hemolysis. Plasma or serum must be separated from the red blood cells as soon as possible. Contamination by ammonia or its salts must be avoided.

TEST PROCEDURE

Special Apparatus

Glass-stoppered centrifuge tubes, 15 ml.
Ostwald-Folin pipettes accurately calibrated "to contain" 0.5 ml.
Capillary pipette, Wintrobe type.
Fister digestion tubes—Pyrex; 25 × 200 mm; glass-stoppered; the 20 ml graduation must be accurately calibrated. These tubes prevent contamination during mixing as well as protect the hands from the action of the alkali. Folin-Wu tubes may be used provided that they are accurately calibrated at 20 ml.
Micro burner, Argand type.
Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.

Reagents

1. **Water, Distilled, Ammonia-Free.** In an all-glass Pyrex distillation apparatus place 1 gm sodium carbonate (Na_2CO_3—Baker & Adamson, A.C.S.) and 1 gm potassium permanganate ($KMnO_4$—Baker & Adamson, A.C.S.); add 500 ml distilled water; redistil, rejecting the first 100 ml portion and collecting only the next 300 ml. Use for the preparation of all reagents and standards and where indicated in the test procedure.
2. **Sodium Sulfate, 23% Aqueous.** Heat approximately 200 ml ammonia-free distilled water (1) to 37° C.; half fill a 100 ml volumetric flask with the warm water; add 23 gm anhydrous sodium sulfate (Na_2SO_4—Baker & Adamson, Reagent Special); add more warm water until the body of the flask is just about filled; stopper and shake until solution is complete; dilute to volume with the warm water. Keep in incubator at 37° C.

   Note: Reagent grade sodium sulfate may vary considerably in its free acid or free alkali content. Each new lot should be tested as follows: dissolve 5 gm in 60 ml carbon dioxide-free distilled water and add 3 drops phenolphthalein indicator solution—no pink color should appear; add 0.5 ml 0.02 N sodium hydroxide—a pink color should appear. Sodium sulfate which does not show these characteristics is unsatisfactory for the differential precipitation of proteins and should be discarded—the reaction of the final solution must be just on the acid side of neutrality.
3. **Ether, Anhydrous** ($(C_2H_5)_2O$—Mallinckrodt, A.C.S.).
4. **Selenium-Sulfuric Acid Digestion Mixture "C" (Fister).** In a 1000 ml Florence flask place 450 ml ammonia-free distilled water (1); add slowly, while cooling the flask under running tap water, 17.6 ml concentrated sulfuric acid (H_2SO_4—Grasselli, A.C.S.); cool to room temperature; transfer to a 500 ml mixing cylinder; add 0.15 ml selenium oxychloride ($SeOCl_2$—J. T. Baker); dilute to volume with ammonia-free distilled water and mix thoroughly. Preserve in Pyrex glass-stoppered bottle. Note: This is not the same digestion mixture used in the nonprotein nitrogen and total nitrogen determinations.
5. **Hydrogen Peroxide, 30%** (H_2O_2—Merck, Reagent Superoxol, Low-Nitrogen).
6. **Nessler Reagent (Folin-Wu).** In a 1000 ml mixing cylinder place 700 ml 2.5 N sodium hydroxide (a); add 150 ml double iodide solution (b); dilute to volume with ammonia-free distilled water (1) and mix; allow to stand for three days to permit sedimentation of the small amount of precipitate which forms. Preserve in ceresin-lined bottle. Stability indefinite. Note: The alkaline concentration of this reagent is 7%—11.0–11.5 ml must be required to neutralize 20 ml 1 N hydrochloric acid, using phenolphthalein as indicator.
   (a) Sodium hydroxide, volumetric 2.5 N; prepare by diluting slightly more than the required amount of concentrated carbonate-free sodium hydroxide solution (see Ap-

pendix); dilute 20 ml of this approximately 2.5 N solution to 100 ml; titrate against standard 0.5 N acid using phenolphthalein as indicator; adjust accordingly and retitrate—20 ml of 0.5 N acid require 20 ml of a 1:5 dilution of 2.5 N alkali. Preserve in ceresin-lined bottle.

(b) Double iodide solution (Folin-Wu); in a 500 ml Florence flask place 150 gm granular potassium iodide (KI—Mallinckrodt, A.C.S.) and 100 gm resublimed iodine (I—Baker & Adamson, A.C.S.); add 100 ml ammonia-free distilled water (1) and shake until the iodine has completely dissolved; add 150 gm redistilled metallic mercury (Hg—Baker & Adamson, A.C.S); shake the flask continuously and vigorously until the red color of the iodine solution has become visibly pale, though still red; immediately start cooling under running tap water, continuing vigorous shaking, until the reddish color has been replaced by the greenish-yellow color of the double iodide. If properly prepared, the solution is clear and there is a residue of shining metallic mercury; solutions showing a gray-green sludge are unsatisfactory. The whole operation should consume no more than 10–15 minutes. Since the reaction develops considerable heat, precautions should be taken to protect the hands during the shaking procedure. Decant the solution into a 2000 ml mixing cylinder, washing mercury and flask with liberal quantities of ammonia-free distilled water and decanting the washings into the cylinder. Dilute to volume with ammonia-free distilled water.

Technic

Note: If globulin is to be estimated by actual determination (Method G-16) instead of by calculation, reserve the precipitate in Step 7.

1. Place a 15 ml glass-stoppered centrifuge tube in a 150 ml beaker half-filled with warm water at 37° C.
2. Without removing the tube from the beaker, add 7.5 ml 23% sodium sulfate (2), removed from the incubator immediately before use.
3. Add 0.5 ml sample from a warm Ostwald-Folin pipette, thoroughly rinsing out the pipette by drawing in and expelling some of the mixture several times.
4. Stopper the tube and mix the contents by inversion.
5. Add 4 ml ether (3) and shake vigorously for 30 seconds.
6. Centrifuge the unstoppered tube at 2000 rpm for 15 minutes—the precipitated globulin is tightly packed in a layer at the interface between the sulfate solution and the ether.
7. Slant the tube so that the globulin layer is separated from the walls; remove about 2 ml of the sulfate layer—this is best done by inserting a Wintrobe-type capillary pipette through the ether layer along the lower wall of the tube into the sulfate layer, holding the index finger over the top of the pipette to prevent the ingress of any ether—RESERVE PRECIPITATE FOR GLOBULIN ANALYSIS (Method G-16).
8. In a 25 ml volumetric flask place
   20 ml ammonia-free distilled water (1)
   1 ml of the sulfate layer from Step 7, carefully wiping the pipette clean of any adherent protein particles.
9. Dilute to volume with ammonia-free distilled water (1) and mix.
10. In a special dry digestion tube (sample) place
    1 ml selenium-sulfuric acid digestion mixture "C" (4)
    1 ml diluted sulfate layer from Step 9
    1 solid glass bead.
11. Clamp tube in a vertical position on a support and apply gentle heat from an Argand micro burner in such a manner that the flame at no time touches the tube above the level of the liquid.

Note: Carry out Steps 11–17 inclusive in a fume hood.

12. After boiling has started gradually increase the heat and place burner in upright position so that the top is about $^3/_4$ inch below the bottom of the tube and the point of the flame directly under the tube—the bubbles should at no time rise above the middle of the tube.
13. Heat until white fumes of SO_3 fill about one-half of the tube—none of these fumes should escape.
14. Insert a small funnel into the mouth of the tube and continue gentle heating with reduced flame until the charred digest begins to clear.
15. Remove the flame and allow to cool for 90 seconds.
16. Remove the funnel and allow 1 drop 30% hydrogen peroxide (5) to fall directly on the digest.
17. Replace funnel and heat gently for 2 minutes making certain that any charred material which may have adhered to the walls of the tube has been washed down and decomposed—the digest should be perfectly clear—if not, add another drop of hydrogen peroxide in the same manner and heat again until clear.
18. Without removing the funnel, allow to cool for 2 minutes and then place the tube in a beaker half-full of ice-cold water.
19. In another special digestion tube (reagent blank) place 1 ml selenium-sulfuric acid digestion mixture "C" (4) and place in the cold water bath with the other tube.
20. After 10 minutes in the cold water bath, add about 8 ml ammonia-free distilled water (1) to each tube, rinsing off the funnel in the sample tube at the same time and mix, allowing the tubes to remain in the bath.

21. Without removing the tubes from the bath add 10 ml Nessler reagent (6), allowing the reagent to fall directly into the solution.
22. Remove the tubes from the bath, dilute to 20 ml with ammonia-free distilled water (1), insert the glass stoppers and mix by inversion.
23. Replace the tubes in the bath and allow to remain for 5 minutes.
24. Remove the tubes from the bath and allow to stand at room temperature (21° C.) for 10 minutes.
25. Measure the transmittancy of the sample against the reagent blank set at 100% transmission at a wave length of 415 mμ and obtain the concentration of nitrogen from the calibration table. Note: This value includes both albumin nitrogen and nonprotein nitrogen.
26. Determine the nonprotein nitrogen concentration on the same sample as outlined in Method N-15a.

Calculation

To determine the albumin concentration—

Total albumin nitrogen-nonprotein nitrogen in mg per 100 ml − Nonprotein nitrogen in mg per 100 ml = Albumin nitrogen in mg per 100 ml

Albumin nitrogen in mg per 100 ml × 0.00625 = Concentration of albumin in per cent

Note: The nonprotein nitrogen correction is seldom made in routine practice.

CALIBRATION PROCEDURE

Special Apparatus

Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.

Fister digestion tubes, glass-stoppered as described in the Test Procedure.

Micro burner, Argand type.

Reagents

1. **Water, Distilled, Ammonia-Free.** Same as for Test Procedure.
4. **Selenium-Sulfuric Acid Digestion Mixture "C" (Fister).** Same as for Test Procedure.
5. **Hydrogen Peroxide, 30%.** Same as for Test Procedure.
6. **Nessler Reagent (Folin-Wu).** Same as for Test Procedure.
7. **Albumin Standards**
    - (a) Nitrogen stock standard (1 ml contains 10 mg nitrogen). Dry pyridine-free ammonium sulfate ($(NH_4)_2SO_4$—Reagent Chemicals, Special) at 110° C. for 1 hour; transfer 4.7166 gm to a 100 ml volumetric flask; add approximately 50 ml ammonia-free distilled water (1); shake until dissolved and dilute to volume with same. Preserve with a few drops of toluene. Stability indefinite.
    - (b) Dilute standard (1 ml contains 0.1 mg nitrogen). In a 100 ml volumetric flask place 1 ml stock standard (7a) and dilute to volume with ammonia-free distilled water (1). Prepare just before use.
    - (c) Calibration standards. In a series of five accurately calibrated 10 ml volumetric flasks place respectively 0, 1, 2, 3 and 4 ml of the dilute standard (7b) and dilute to volume with ammonia-free distilled water (1). These standards represent concentrations of nitrogen equivalent to 0, 400, 800, 1200 and 1600 mg per 100 ml sample. Prepare just before use.

Technic

1. Into correspondingly marked special dry digestion tubes place
    - 1 ml of each calibration standard (7c)
    - 1 ml selenium-sulfuric acid digestion mixture "C" (4)
    - 1 solid glass bead.
2. Treat each tube exactly as outlined in the Test Procedure, Steps 11–24 inclusive—the standards are run consecutively through Steps 11–18 inclusive, Step 19 is omitted (since this duplicates the zero concentration calibration standard) and then simultaneously through Steps 20–24 inclusive.
3. Set the zero concentration standard at 100% transmission at a wave length of 415 mμ and record the transmittancy of the 400, 800, 1200 and 1600 mg per cent standards. The transmittancy of the 1600 mg standard should be approximately 30%.
4. Plot the observed values on semi-log graph paper—the points fall on a very slight curve which passes through the concentration axis slightly to the right of the origin (Calibration Curve A-11).
5. Prepare a calibration table from the T-C values obtained from the plotted curve.

PROCEDURE FOR USE WITH LINEAR-SCALE PHOTOELECTRIC COLORIMETERS (SHEARD-SANFORD TYPE)

TEST PROCEDURE

Exactly as outlined; set the reagent blank at 100 and use a blue filter.

CALIBRATION PROCEDURE

Exactly as outlined—use a blue filter; plot the observed readings on semi-log graph paper—the points fall on a slight curve passing through the concentration axis slightly to the right of the origin.

PROCEDURE FOR USE WITH LOGARITHMIC-SCALE PHOTOELECTRIC COLORIMETERS (KLETT-SUMMERSON TYPE)

TEST PROCEDURE

Exactly as outlined; set the reagent blank at 0 and use a blue filter.

CALIBRATION PROCEDURE

Exactly as outlined—use a blue filter; plot the observed readings on linear graph paper—the points fall on a slight curve passing through the concentration axis slightly to the right of the origin.

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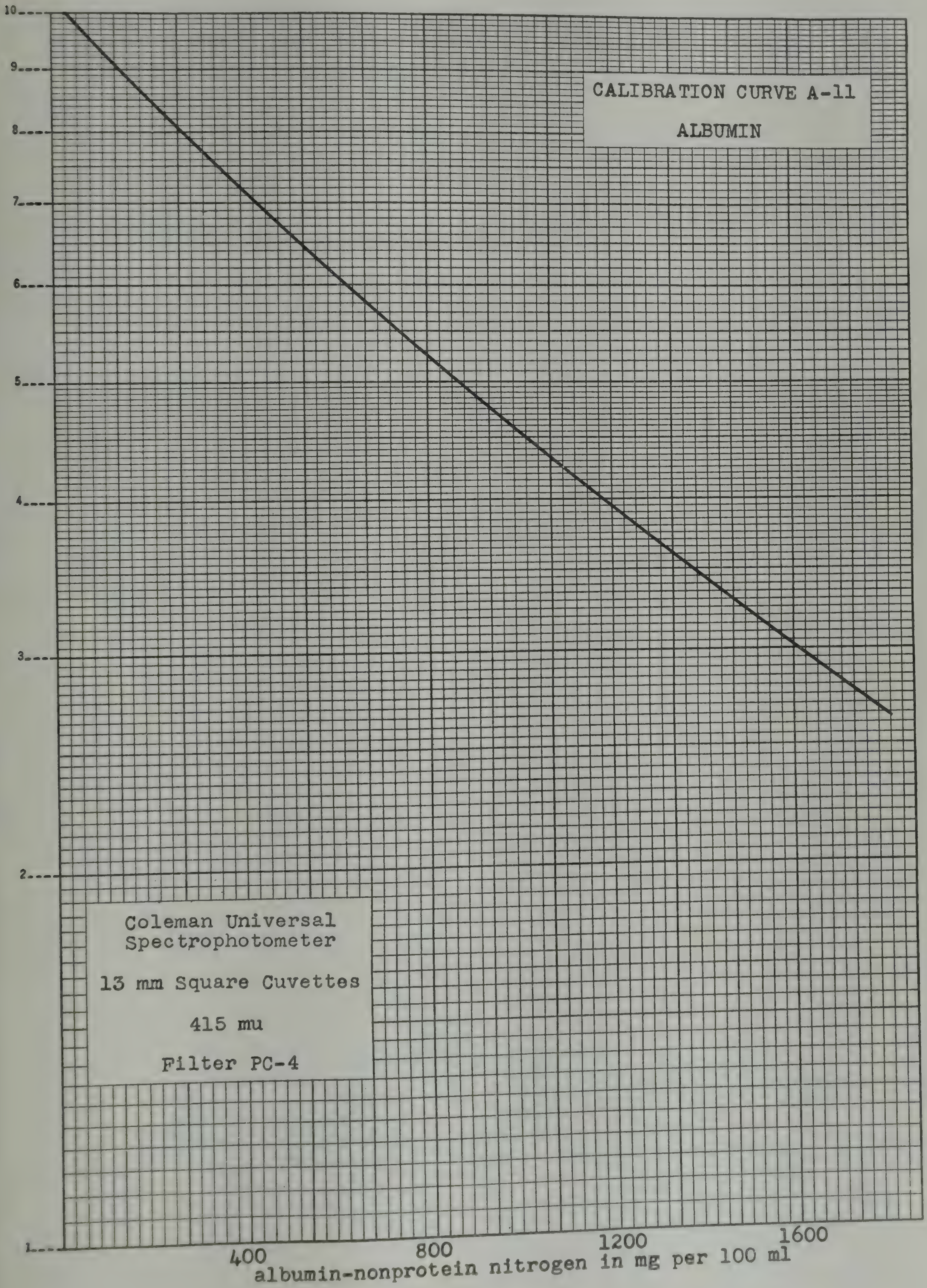
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CALIBRATION CURVE A-11
ALBUMIN
Coleman Universal Spectrophotometer
13 mm Square Cuvettes
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Filter PC-4
400
800
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1600
albumin-nonprotein nitrogen in mg per 100 ml

Meter Reading	0	1	2	3	4	5	6	7	8	9
0										
10										
20										
30										
40										
50										
60										
70										
80										
90										

ALBUMIN

A spectrophotometric adaptation based on the methods and modifications of Howe (1921), Wu (1922), Wu and Ling (1927), Folin and Ciocalteu (1927), Greenberg (1929), Andersch and Gibson (1933), Greenberg and Mirolubova (1936), Kingsley (1940) and Maclay and Osterberg (1940)

PRINCIPLE

Albumin is separated from globulin by precipitation of the latter with sodium sulfate, ether being used to reduce the density of the precipitated globulin thus permitting the final separation to be made by centrifugation. The albumin in the globulin-free centrifugate is then determined by means of the Folin-Ciocalteu phenol reagent as outlined in the method for total protein (Method P-52), the same analytical principles being involved. One milligram tyrosine produces a color equivalent to the color produced by 10.5 mg albumin.

DETERMINATION IN PLASMA OR SERUM

SAMPLE

Plasma or serum from blood withdrawn during postabsorptive state. Tourniquet must be removed immediately after entering vein and collection of blood should be completed within one minute. Anticoagulant—potassium oxalate. There must be no trace of hemolysis. Plasma or serum must be separated from the red blood cells as soon as possible.

TEST PROCEDURE

Special Apparatus

Glass-stoppered centrifuge tubes, 15 ml.

Ostwald-Folin pipettes accurately calibrated "to contain" 0.5 ml.

Capillary pipette, Wintrobe type.

Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.

Reagents

1. **Sodium Sulfate, 23% Aqueous.** Heat approximately 200 ml distilled water to 37° C.; half fill a 100 ml volumetric flask with the warm water; add 23 gm anhydrous sodium sulfate (Na_2SO_4—Baker & Adamson, Reagent Special); add more warm water until the body of the flask is just about filled; stopper and shake until solution is complete; dilute to volume with the warm water. Keep in incubator at 37° C.

   Note: Reagent grade sodium sulfate may vary considerably in its free acid or free alkali content. Each new lot should be tested as follows: dissolve 5 gm in 60 ml carbon dioxide-free distilled water and add 3 drops phenolphthalein indicator solution—no pink color should appear; add 0.5 ml 0.02 N sodium hydroxide—a pink color should appear. Sodium sulfate which does not show these characteristics is unsatisfactory for the differential precipitation of proteins and should be discarded—the reaction of the final solution must be just on the acid side of neutrality.

2. **Ether, Anhydrous** ($(C_2H_5)_2O$—Mallinckrodt, A.C.S.).
3. **Sodium Hydroxide, Volumetric 2.5 N.** Prepare by diluting slightly more than the required amount of concentrated carbonate-free sodium hydroxide solution (see Appendix); dilute 20 ml of this approximately 2.5 N solution to 100 ml; titrate against standard 0.5 N acid using phenolphthalein as indicator; adjust accordingly and re-titrate—20 ml of 0.5 N acid require 20 ml of a 1:5 dilution of 2.5 N alkali. Preserve in ceresin-lined bottle.
4. **Phenol Reagent (Folin-Ciocalteu).** In a 1500 ml boiling flask with ground joint place 500 ml distilled water; add 100 gm sodium tungstate ($Na_2WO_4 \cdot 2H_2O$—Mallinckrodt, Folin Special) and shake until dissolved; add 25 gm sodium molybdate ($Na_2MoO_4 \cdot 2H_2O$—Mallinckrodt, AR); add 200 ml distilled water, washing down the neck of the flask and shake until solution is complete; add 50 ml 85% phosphoric acid (H_3PO_4—Baker & Adamson, A.C.S.) and mix thoroughly; add 100 ml concentrated hydrochloric acid (HCl—Baker & Adamson, A.C.S.) and mix thoroughly; fit a Liebig reflux condenser to the flask and boil gently for 10 hours (during this period the solution changes in color from a pale yellow to a deep green); remove the reflux condenser; add 150 gm lithium sulfate ($LiSO_4$—Baker & Adamson, Reagent) and 50 ml distilled water, washing down the neck of the flask; shake thoroughly until completely dissolved; add 10 drops bromine (Br—Baker & Adamson, A.C.S.) and boil for 15 minutes to remove the excess bromine; allow to cool to room temperature; dilute to 1000 ml with distilled water and filter through tightly packed glass wool into a brown glass-stoppered bottle. The reagent should show no tint of green and if kept well protected from dust will remain stable for months. A reagent which develops a green color in the course of time may be repurified by the addition of a few drops of bromine and boiling as before.

Technic

Note: If globulin is to be estimated by actual determination (Method G-17) instead of by calculation, reserve the precipitate in Step 7.

1. Place a 15 ml glass-stoppered centrifuge tube in a 150 ml beaker half-filled with warm water at 37° C.
2. Without removing the tube from the beaker, add 7.5 ml 23% sodium sulfate (1), removed from the incubator immediately before use.
3. Add 0.5 ml sample from a warm Ostwald-Folin pipette, thoroughly rinsing out the pipette by

drawing in and expelling some of the mixture several times.

4. Stopper the tube and mix the contents by inversion.
5. Add 4 ml ether (2) and shake vigorously for 30 seconds.
6. Centrifuge the unstoppered tube at 2000 rpm for 15 minutes—the precipitated globulin is tightly packed in a layer at the interface between the sulfate solution and the ether.
7. Slant the tube so that the globulin layer is separated from the walls; remove about 2 ml of the sulfate layer—this is best done by inserting a Wintrobe-type capillary pipette through the ether layer along the lower wall of the tube into the sulfate layer, holding the index finger over the top of the pipette to prevent the ingress of any ether—RESERVE PRECIPITATE FOR GLOBULIN ANALYSIS (Method G-17).
8. In an 18 × 150 mm Pyrex test tube place
   4.5 ml distilled water
   1.5 ml of the sulfate layer from Step 7, carefully wiping the pipette clean of any adherent protein particles
   4 ml 2.5 N sodium hydroxide (3) and mix.
9. Place in boiling water for 10 minutes.
10. Cool in running tap water for 3 minutes.
11. Quantitatively transfer, through a funnel and Whatman #40 paper into a 100 ml volumetric flask (sample)—the filtration should be carried out even if no prepicitate is apparent.
12. In another 100 ml volumetric flask (reagent blank) place 4 ml 2.5 N sodium hydroxide (3).
13. To each flask add about 50 ml distilled water and mix.
14. Add 3 ml phenol reagent (4), slowly and with constant agitation of the flasks.
15. Dilute to volume with distilled water and mix thoroughly by inversion.
16. Allow to stand at room temperature for 10 minutes.
17. Measure the transmittancy of the sample against the reagent blank set at 100% transmission at a wave length of **700 mμ** and multiply the value from the calibration table for total protein (Method P-52) by 0.485 to obtain the concentration of albumin in per cent. If a calibration table has been prepared for albumin, the concentration is obtained directly.

CALIBRATION PROCEDURE

Note: The following calibration procedure is optional. The values in the calibration table for total protein (Method P-52), when multiplied by 0.485, are equivalent to the concentration of albumin in per cent.

Special Apparatus

Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.

Reagents

3. **Sodium Hydroxide, Volumetric 2.5 N.** Same as for Test Procedure.
4. **Phenol Reagent (Folin-Ciocalteu).** Same as for Test Procedure.
5. **Hydrochloric Acid, Volumetric 0.1 N.** Add 10 ml concentrated hydrochloric acid (HCl—Baker & Adamson, A.C.S.) to 1000 ml distilled water and mix; standardize against 0.1 N alkali using phenolphthalein as indicator.
6. **Albumin Standards**
   (a) Tyrosine stock standard (1 ml contains 0.2 mg tyrosine). In a 100 ml volumetric flask place 20 mg l-tyrosine ($C_9H_{11}O_3N$—Pfanstiehl, CP), add about 85 ml 0.1 N hydrochloric acid (5); shake until dissolved and dilute to volume with same. Preserve with a few drops of xylene. Store in refrigerator.
   (b) Dilute standard (1 ml contains 0.05 mg tyrosine). In a 100 ml volumetric flask place 25 ml stock standard (6a) and dilute to volume with distilled water. Prepare just before use.
   (c) Calibration standards. In a series of six accurately calibrated 100 ml volumetric flasks place respectively 0, 2.5, 5, 7.5, 10 and 12.5 ml of the dilute standard (6b) and proceed as follows. These standards represent concentrations of albumin equivalent to 0, 1.4, 2.8, 4.2, 5.6 and 7.0%. Prepare just before use.

Technic

1. To each calibration standard (6c) add 4 ml 2.5 N sodium hydroxide (3).
2. Proceed exactly as outlined in the Test Procedure, Steps 13–16 inclusive.
3. Set the zero concentration standard at 100% transmission at a wave length of **700 mμ** and record the transmittancy of the 1.4, 2.8, 4.2, 5.6 and 7.0% standards. The transmittancy of the 7.0% standard should be approximately 27%.
4. Plot the observed values on semi-log graph paper —the points fall on a straight line which passes through the origin (Calibration Curve A-12).
5. Prepare a calibration table from the T-C values obtained from the plotted curve.

PROCEDURE FOR USE WITH LINEAR-SCALE PHOTOELECTRIC COLORIMETERS (SHEARD-SANFORD TYPE)

TEST PROCEDURE

Exactly as outlined; set the reagent blank at 100 and use a green filter.

CALIBRATION PROCEDURE

Exactly as outlined—use a green filter; plot the observed readings on semi-log graph paper—the points fall on a straight line passing through the origin.

PROCEDURE FOR USE WITH LOGARITHMIC-SCALE PHOTOELECTRIC COLORIMETERS (KLETT-SUMMERSON TYPE)

TEST PROCEDURE

Exactly as outlined; set the reagent blank at 0 and use a green filter.

CALIBRATION PROCEDURE

Exactly as outlined—use a green filter; plot the observed readings on linear graph paper—the points fall on a straight line passing through the origin.

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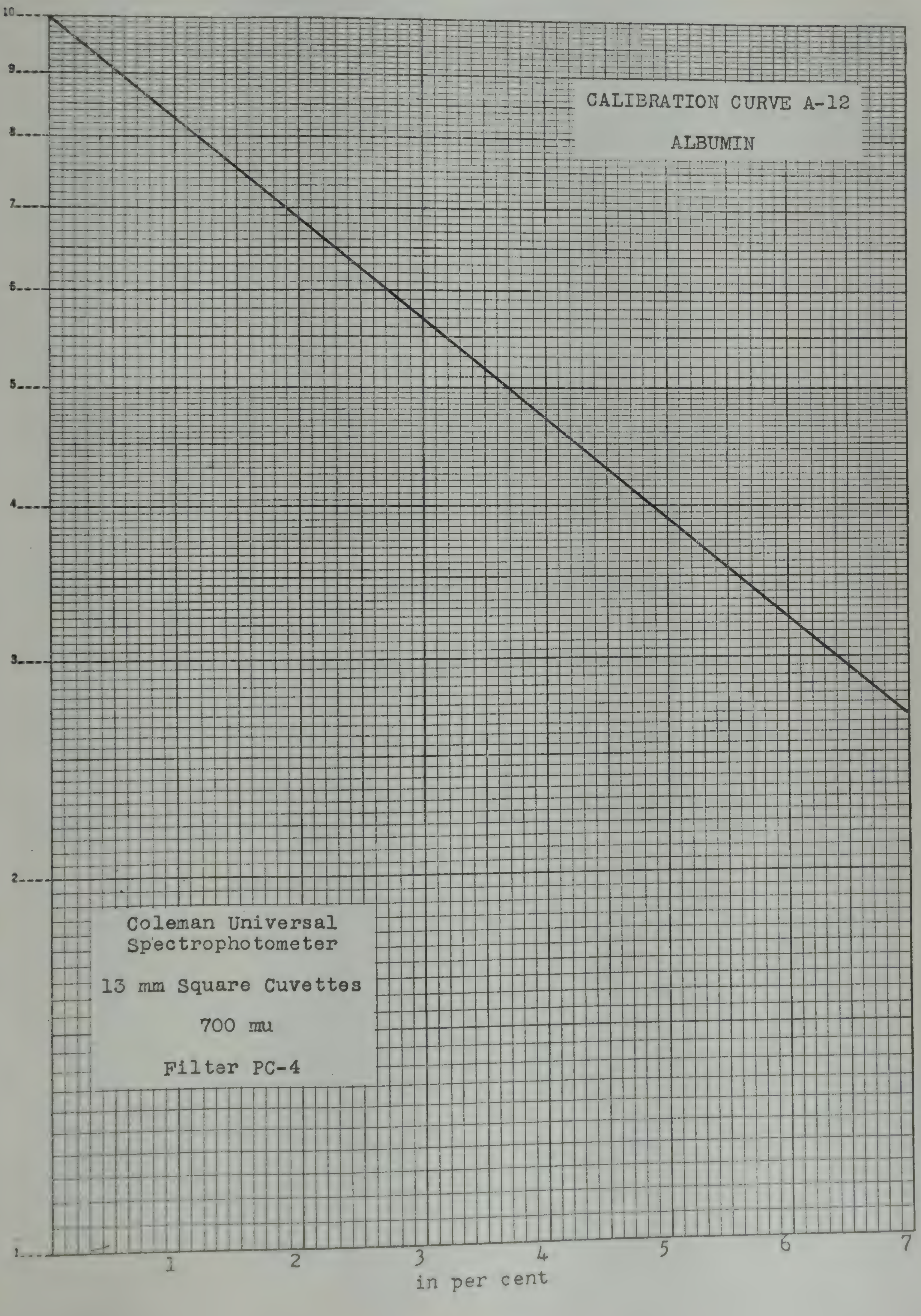
NOTES

NOTES


CALIBRATION CURVE A-12
ALBUMIN
Coleman Universal
Spectrophotometer
13 mm Square Cuvettes
700 mu
Filter PC-4
10
9
8
7
6
5
4
3
2
1
1
2
3
4
5
6
7
in per cent

Meter Reading	0	1	2	3	4	5	6	7	8	9
0										
10										
20										
30										
40										
50										
60										
70										
80										
90										

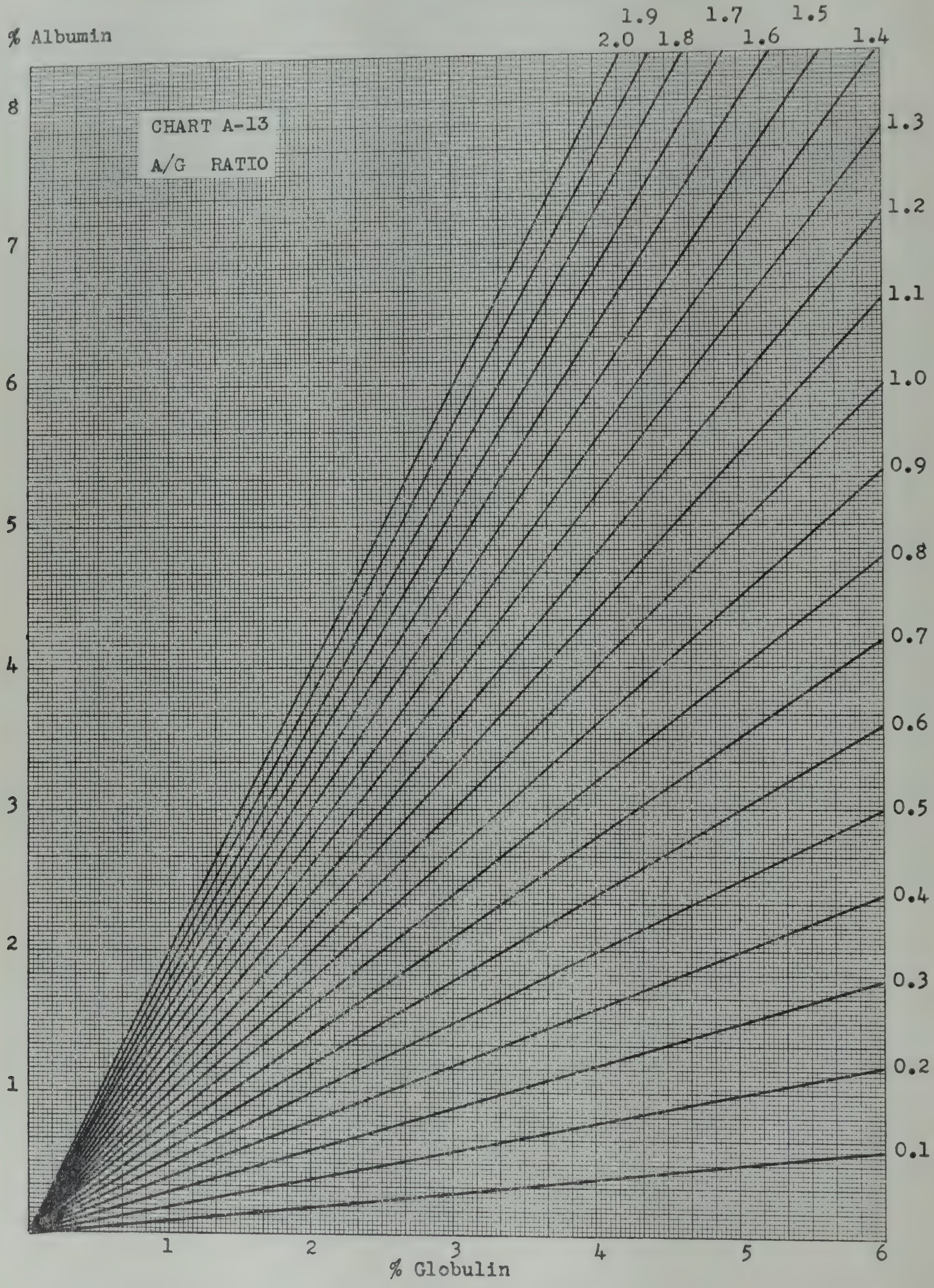
ALBUMIN:GLOBULIN RATIO

1. Determine albumin by any of the outlined methods.
2. Determine globulin by any of the outlined methods.
3. Calculation—
   Albumin in % ÷ Globulin in % = A/G Ratio

Note: The ratio may be obtained directly from Chart A-13. For maximum accuracy, the same type of procedure should be used for determining both albumin and globulin.

Locate the determined percentage of albumin on the vertical axis of the chart; move horizontally across the chart to the determined percentage of globulin and then along the nearest diagonal line to the margin to obtain the A/G ratio.

NOTES


% Albumin
CHART A-13
A/G RATIO
1.9
1.7
1.5
2.0
1.8
1.6
1.4
1.3
1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
8
7
6
5
4
3
2
1
1
2
3
4
5
6
% Globulin

AMINO ACID NITROGEN

A spectrophotometric adaptation based on the methods and modifications of
Kober and Sugiura (1912; 1913), Kober (1917), Pope and Stevens (1939),
Krauel (1944), Albanese and Irby (1944; 1945) and Fister (1948)

PRINCIPLE

Amino acids, in alkaline solution, are treated with copper phosphate to form soluble cupric salts of the various amino acids (Kober-Sugiura reaction). After removal of the excess phosphate, the copper of the soluble amino acid salts is determined indirectly by the color of the iodine liberated from an excess of potassium iodide in the presence of phosphoric acid.

DETERMINATION IN WHOLE BLOOD, PLASMA OR SERUM

SAMPLE

Blood withdrawn during post-absorptive state. Anticoagulant—potassium oxalate; lithium oxalate preferable. Analyses preferably made on plasma or serum; hemolysis must be avoided. Determinations should be carried out as soon as possible after collection of sample. Ammonia and other nonprotein nitrogenous constituents do not interfere and need not be removed from the sample.

TEST PROCEDURE

Special Apparatus

Ostwald-Folin pipettes accurately calibrated "to deliver" 2 ml.

Graduated centrifuge tubes—15 ml in 0.1 ml; the 10 ml graduation must be accurately calibrated.

Capillary droppers delivering 40 drops per ml.

Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.

Reagents

1. **Trichloracetic Acid, 10% Aqueous.** Dilute 20 ml concentrated trichloracetic acid (a) to 200 ml with distilled water. Prepare fresh weekly.
    - (a) Trichloracetic acid, concentrated; dissolve 100 gm crystalline trichloracetic acid (CCl_3COOH—J. T. Baker, CP Analyzed) in distilled water and dilute to 100 ml. Preserve in glass-stoppered bottle. Stability indefinite.
2. **Trichloracetic Acid, 7.5% Aqueous.** Dilute 75 ml 10% trichloracetic acid (1) to 100 ml with distilled water. Prepare fresh weekly.
3. **Thymolphthalein Indicator Solution, 0.25% Alcoholic.** Dissolve 250 mg thymolphthalein ($C_{28}H_{30}O_4$—National Aniline) in 75 ml absolute methyl alcohol (CH_3OH—Merck, A.C.S.) and dilute to 100 ml with distilled water.
4. **Sodium Hydroxide, Volumetric 2.5 N.** Prepare by diluting slightly more than the required amount of concentrated carbonate-free sodium hydroxide solution (see Appendix); dilute 20 ml of this approximately 2.5 N solution to 100 ml; titrate against standard 0.5 N acid using phenolphthalein as indicator; adjust accordingly and re-titrate—20 ml of 0.5 N acid require 20 ml of a 1:5 dilution of 2.5 N alkali. Preserve in ceresin-lined bottle.
5. **Copper Phosphate Suspension (Pope-Stevens).** To two volumes of 0.18 M tribasic sodium phosphate (a) add one volume of 0.16 M cupric chloride (b) and mix; slowly and with shaking, add two volumes of borate buffer solution (c); mix thoroughly. Preserve in refrigerator. Stability approximately one week.
    - (a) Sodium phosphate, tribasic, 0.18 M; dissolve 7.17 gm tribasic sodium phosphate ($Na_3PO_4 \cdot 12H_2O$—Merck, Reagent) in distilled water and dilute to 100 ml. Preserve in ceresin-lined bottle.
    - (b) Cupric chloride, 0.16 M; dissolve 2.73 gm crystalline cupric chloride ($CuCl_2 \cdot 2H_2O$—Baker & Adamson, Reagent) in distilled water and dilute to 100 ml.
    - (c) Borate buffer solution (Pope-Stevens); in a 200 ml volumetric flask place 5.721 gm sodium tetraborate ($Na_2B_4O_7 \cdot 10H_2O$—Baker & Adamson, A.C.S.); add approximately 150 ml distilled water and shake until dissolved; add 10 ml 1 N hydrochloric acid (i); dilute to volume with distilled water and mix. Filter into a resistant-glass bottle.
        - (i) Hydrochloric acid, volumetric 1 N; add 100 ml concentrated hydrochloric acid (HCl—Baker & Adamson, A.C.S.) to 1000 ml distilled water and mix; standardize against 1 N alkali using phenolphthalein as indicator.
6. **Phosphoric acid, Volumetric 0.5 N.** Dilute 20 ml 85% phosphoric acid (H_3PO_4—Baker & Adamson, A.C.S.) to 1000 ml with distilled water and mix; titrate against standard 0.1 N alkali using phenolphthalein as indicator; adjust accordingly and re-titrate—5 ml of 0.5 N acid require 25 ml of 0.1 N alkali.
7. **Potassium Iodide Solution.** Dissolve 1 gm crystalline potassium iodide (KI—J. T. Baker, Low-Iodate, Special) per ml of distilled water. Prepare only the amount required just before use; 3 ml are sufficient for one determination including blank.

Technic

Note: For concentrations of amino acid nitrogen in excess of 12 mg per 100 ml, dilute 2 ml of the protein-free filtrate in Step 5 with an equal volume of 7.5% trichloracetic acid (2) and multiply the values obtained from the calibration table by 2.

1. In a 15 ml centrifuge tube place
   6 ml 10% trichloracetic acid (1)
   2 ml sample from an Ostwald-Folin pipette, added drop by drop, with constant agitation of the tube.
2. Mix thoroughly and allow to stand for 10 minutes.
3. Centrifuge at 1500 rpm for 3 minutes.
4. Decant supernatant fluid into a small dry test tube through a dry 2.5 cm funnel with a Whatman #5 filter paper cut to fit.
5. In a 15 ml graduated centrifuge tube (sample) place
   4 ml filtrate.
6. In another 15 ml graduated centrifuge tube (reagent blank) place
   4 ml 7.5% trichloracetic acid (2).
7. To each tube add, from a capillary-tipped dropper, 1 drop thymolphthalein indicator solution (3) and mix.
8. Add, from a capillary-tipped dropper, 2.5 N sodium hydroxide (4), drop by drop and with constant shaking, until a permanent blue color appears (about 25–27 drops are usually necessary).
9. Add 5 ml thoroughly mixed copper phosphate suspension (5).
10. Adjust volume to 10 ml with distilled water and shake vigorously—the color is an opaque greenish blue.
11. Allow to stand for 5 minutes.
12. Centrifuge at 1500 rpm for 5 minutes.
13. Carefully transfer 5 ml of each crystal-clear supernatant fluid to a correspondingly marked cuvette—if the supernatant fluid is not colorless, but tinged with blue, too much sodium hydroxide was added in Step 8 and the analysis must be repeated.
14. Add 1 ml 0.5 N phosphoric acid (6) and mix.
15. Add 1 ml freshly prepared potassium iodide solution (7) and mix—the color becomes a pale yellow.
16. Add 3 ml distilled water and mix.
17. Place in water bath at 25°C. for 5 minutes.
18. Measure the transmittancy of the sample against the reagent blank set at 100% transmission at a wave length of 400 mμ and obtain the concentration of amino acid nitrogen from the calibration table.

CALIBRATION PROCEDURE

Special Apparatus

Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.

Graduated test tubes—10 ml in 0.1 ml; the 8 ml graduation must be accurately calibrated.

Graduated centrifuge tubes—15 ml in 0.1 ml; the 10 ml graduation must be accurately calibrated.

Capillary droppers delivering 40 drops per ml.

Reagents

2. **Trichloracetic Acid, 7.5% Aqueous.** Same as for Test Procedure.
3. **Thymolphthalein Indicator Solution.** Same as for Test Procedure.
4. **Sodium Hydroxide, Volumetric 2.5 N.** Same as for Test Procedure.
5. **Copper Phosphate Suspension (Pope-Stevens).** Same as for Test Procedure.
6. **Phosphoric Acid, Volumetric 0.5 N.** Same as for Test Procedure.
7. **Potassium Iodide Solution.** Same as for Test Procedure.
8. **Hydrochloric Acid-Benzoate Solution.** In a 1000 ml volumetric flask place 2 gm sodium benzoate ($NaC_7H_5O_2$—Merck, U.S.P.); add approximately 800 ml distilled water and shake until dissolved; add 70 ml 1 N hydrochloric acid (5i) dilute to volume with distilled water. Filter, if necessary, before use.
9. **Amino Acid Standards**
   (a) Aminoacetic acid stock standard (1 ml contains 0.1 mg amino acid nitrogen). Dry aminoacetic acid ($C_2H_5O_2N$—Reagent Chemicals, Ammonia-Free Special) over sulfuric acid in vacuo to constant weight; transfer 267.5 mg to a 500 ml volumetric flask; add approximately 400 ml hydrochloric acid-benzoate solution (8); shake until dissolved and dilute to volume with same. Stability indefinite.
   (b) Proline stock standard (1 ml contains 0.1 mg amino acid nitrogen). Dry l(-)proline ($C_5H_9O_2N$—Merck) over sulfuric acid in vacuo to constant weight; transfer 187.8 mg to a 500 ml volumetric flask; add approximately 400 ml hydrochloric acid-benzoate solution (8); shake until dissolved and dilute to volume with same. Stability indefinite.
   (c) Dilute standard (1 ml contains 0.03 mg amino acid nitrogen). In a 100 ml volumetric flask place 15 ml aminoacetic acid stock standard (9a) and 15 ml proline stock standard (9b); add 20 ml distilled water and 7.5 ml concentrated trichloracetic acid (1a) and mix; dilute to volume with distilled water. Preserve in refrigerator. Stability about one week.
   (d) Calibration standards. In a series of six accurately calibrated 10 ml test tubes place respectively 0, 1, 2, 4, 6 and 8 ml of the dilute standard (9c); add 8, 7, 6, 4, 2 and 0

ml 7.5% trichloracetic acid (2) respectively bringing the volume in each tube to 8 ml. These standards represent concentrations of amino acid nitrogen equivalent to 0, 1.5, 3.0, 6.0, 9.0 and 12 mg per 100 ml sample. Prepare just before use.

Technic

1. Transfer 4 ml of each calibration standard (9d) to a correspondingly marked 15 ml graduated centrifuge tube.
2. To all tubes add, from a capillary-tipped dropper, 1 drop thymolphthalein indicator solution (3) and mix.
3. Proceed exactly as outlined in the Test Procedure, Steps 8–17 inclusive.
4. Set the zero concentration standard, which should be practically colorless, at 100% transmission at a wave length of **400** mμ and record the transmittancy of the 1.5, 3.0, 6.0, 9.0 and 12 mg per cent standards. The transmittancy of the 12 mg standard should be approximately 24%.
5. Plot the observed values on semi-log graph paper—the points fall on a smooth curve which passes through the origin (Calibration Curve A-15).
6. Prepare a calibration table from the T-C values obtained from the plotted curve.

Method A-15b

DETERMINATION IN URINE

SAMPLE

Aliquot of a 24-hour specimen collected in a clean, dry, brown glass, two-quart bottle containing 50 ml 15% hydrochloric acid (10) and 1 ml 10% thymol (11). Place each voiding as passed into this bottle. The bottle need not be kept in the refrigerator. Amino acid nitrogen values remain constant for at least one week. Ammonia need not be removed.

TEST PROCEDURE

Special Apparatus

As listed under the Test Procedure for blood.

Reagents

1. **Trichloracetic Acid, 10% Aqueous.** Same as for blood.
2. **Trichloracetic Acid, 7.5% Aqueous.** Same as for blood.
3. **Thymolphthalein Indicator Solution.** Same as for blood.
4. **Sodium Hydroxide, Volumetric 2.5 N.** Same as for blood.
5. **Copper Phosphate Suspension (Pope-Stevens).** Same as for blood.
6. **Phosphoric Acid, Volumetric 0.5 N.** Same as for blood.
7. **Potassium Iodide Solution.** Same as for blood.

10. **Hydrochloric Acid, 15% by Volume.** Dilute 15 ml concentrated hydrochloric acid (HCl—Baker & Adamson, A.C.S.) to 100 ml with distilled water.
11. **Thymol, 10% Alcoholic.** Dissolve 10 gm crystalline thymol ($C_{10}H_{14}O$—Merck, Reagent) in 100 ml absolute methyl alcohol (CH_3OH—Merck, A.C.S.).

Technic

Note: For concentrations of amino acid nitrogen in excess of 24 mg per 100 ml, dilute 1 ml or 2 ml of filtered urine to 10 ml in Step 4 and multiply the values obtained from the calibration table for blood by 10 or 5 respectively.

1. Transfer the entire contents of the collection bottle, if less than a liter to a 1000 ml mixing cylinder and if more than a liter to a 2000 ml mixing cylinder; note the volume and subtract 51 ml (preservatives added)—record this value as the total 24-hour amount.
2. Dilute to volume (1000 ml or 2000 ml) with distilled water and mix thoroughly.
3. Filter approximately 25 ml diluted urine into a dry Erlenmeyer flask.
4. In a 10 ml volumetric flask place 5 ml filtered urine and dilute to volume with distilled water.
5. In a 15 ml centrifuge tube place
    6 ml 10% trichloracetic acid (1)
    2 ml diluted urine from an Ostwald-Folin pipette, added drop by drop, with constant agitation of the tube.
6. Proceed exactly as outlined in the Test Procedure for blood, Steps 2–17, inclusive.
7. Measure the transmittancy of the sample against the reagent blank set at 100% transmission at a wave length of **400** mμ and multiply the value from the calibration table for blood by 2. Correct for the original dilution of urine as follows.

Calculation

To correct for the original dilution of urine in Step 2—

$$\text{Concentration from calibration table} \times \frac{\text{Diluted volume (in Step 2)}}{\text{24-hour volume (in Step 1)}} = \text{Concentration of amino acid nitrogen in mg per 100 ml urine}$$

$$\frac{\text{Volume of 24-hour specimen}}{100} \times \text{Amino acid nitrogen in mg per 100 ml} = \text{Mg amino acid nitrogen excreted in 24 hours}$$

PROCEDURE FOR USE WITH LINEAR-SCALE PHOTOELECTRIC COLORIMETERS (SHEARD-SANFORD TYPE)

TEST PROCEDURE

Exactly as outlined; set the reagent blank at 100 and use a blue filter.

CALIBRATION PROCEDURE

Exactly as outlined—use a blue filter; plot the observed readings on semi-log graph paper—the points fall on a slight curve passing through the origin.

PROCEDURE FOR USE WITH LOGARITHMIC-SCALE PHOTOELECTRIC COLORIMETERS (KLETT-SUMMERSON TYPE)

TEST PROCEDURE

Exactly as outlined; set the reagent blank at 0 and use a blue filter.

CALIBRATION PROCEDURE

Exactly as outlined—use a blue filter; plot the observed readings on linear graph paper—the points fall on a slight curve passing through the origin.

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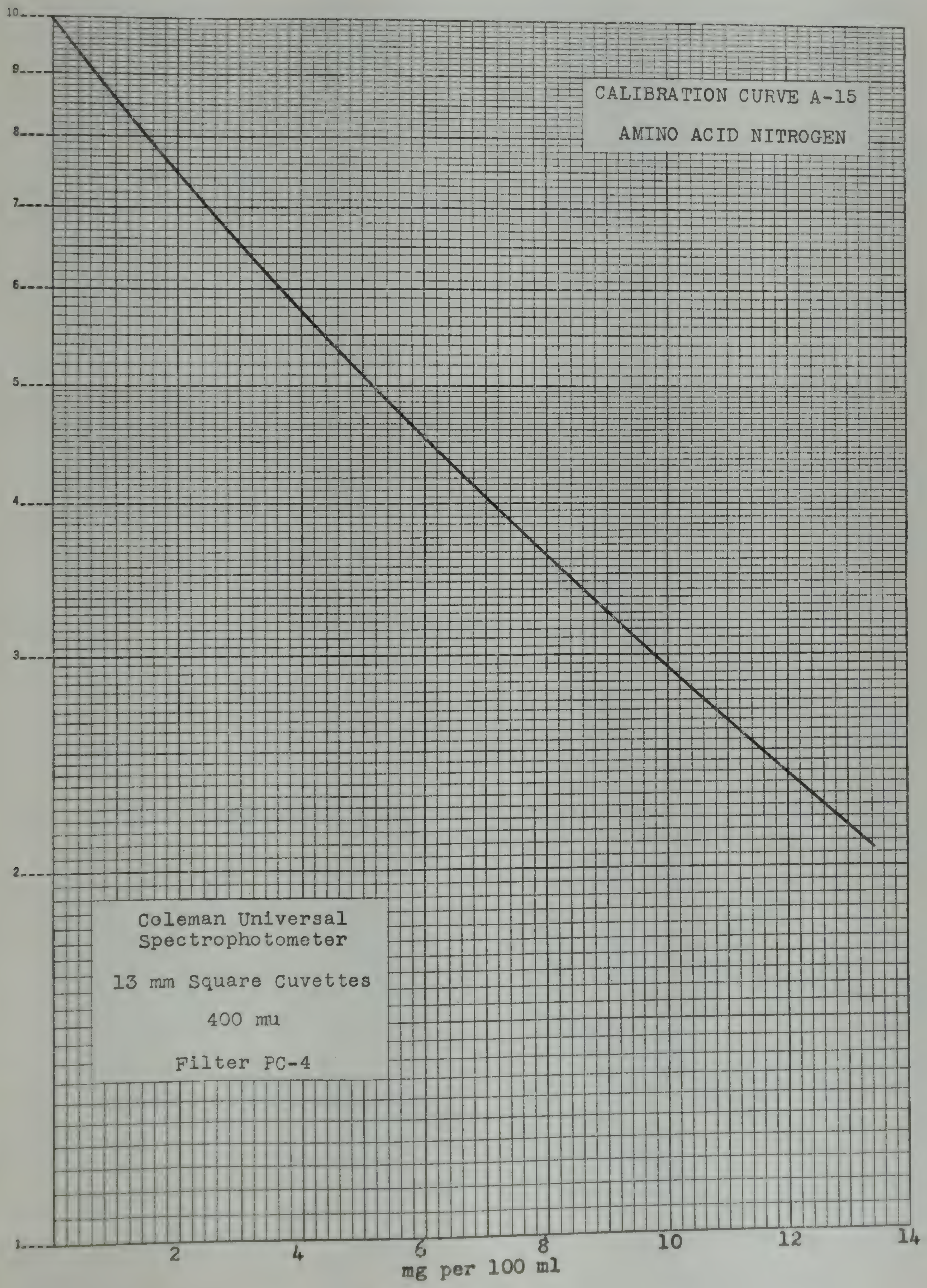
NOTES


10
9
8
7
6
5
4
3
2
1
CALIBRATION CURVE A-15
AMINO ACID NITROGEN
Coleman Universal
Spectrophotometer
13 mm Square Cuvettes
400 mu
Filter PC-4
2
4
6
8
10
12
14
mg per 100 ml

Meter Reading	0	1	2	3	4	5	6	7	8	9
0										
10										
20										
30										
40										
50										
60										
70										
80										
90										

AMINO ACID NITROGEN

A spectrophotometric adaptation based on the methods and modifications of Folin and Wu (1922), Danielson (1933), Sahyun and Goodell (1939), Frame, Russell and Wilhelmi (1943), Krauel (1944) and Russell (1944)

PRINCIPLE

Amino acids in an alkaline medium, the pH of which is carefully controlled, are treated with sodium b-naphthoquinone-4-sulfonate to produce an orange-red color (Folin-Wu reaction). The excess color due to the naphthoquinone is destroyed by sodium thiosulfate, acid formaldehyde being used to prevent the precipitation of free sulfur.

DETERMINATION IN WHOLE BLOOD, PLASMA OR SERUM

SAMPLE

Blood withdrawn during post-absorptive state. Anticoagulant—potassium oxalate; lithium oxalate preferable. Analyses preferably made on plasma or serum; hemolysis must be avoided. Determinations should be carried out as soon as possible after collection of sample. Since ammonia interferes in the color reaction, this method is not applicable to the determination of amino acids in urine.

Note: Uric acid and the sulfonamides also produce color with this method—in the case of the former, a concentration of 1 mg per 100 ml gives a color equivalent to 0.1 mg amino nitrogen, and with the latter, a concentration of 10 mg free sulfonamide per 100 ml gives a color equivalent to 0.8 mg amino nitrogen. When the uric acid concentration in the blood is increased or when sulfonamides have been administered due correction should be made.

TEST PROCEDURE

Special Apparatus

Ostwald-Folin pipettes accurately calibrated "to contain" 1 ml.

Graduated test tubes—25 ml in 0.5 ml; the 25 ml graduation must be accurately calibrated.

Capillary droppers delivering 40 drops per ml.

Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.

Note: Glassware which has been cleaned with dichromate-sulfuric acid solution must be rinsed with an alkaline wash solution to remove all traces of chromic acid, again rinsed with ammonia-free distilled water and oven dried before use.

Reagents

Note: All reagents must be prepared with ammonia-free distilled water (Reagent 1) and should be protected from contamination by ammonia. The sulfuric acid and sodium tungstate, since they are not used in the preparation of the reagent blank, should be tested occasionally for chromogenic materials by comparison with a distilled water blank.

1. **Water, Distilled, Ammonia-Free.** In an all-glass Pyrex distillation apparatus place 1 gm sodium carbonate (Na_2CO_3—Baker & Adamson, A.C.S.) and 1 gm potassium permanganate ($KMnO_4$—Baker & Adamson, A.C.S.); add 500 ml distilled water; redistil, rejecting the first 100 ml portion and collecting only the next 300 ml. Use for the preparation of all reagents and standards and where indicated in the test procedure.
2. **Sulfuric Acid, Volumetric N/12.** Add 2.5 ml concentrated sulfuric acid (H_2SO_4—Grasselli, A.C.S.) to 1000 ml ammonia-free distilled water (1) and mix well; titrate against standard 0.1 N alkali using phenolphthalein as indicator; adjust accordingly and re-titrate—20 ml of N/12 acid require 16.7 ml of 0.1 N alkali.
3. **Sodium Tungstate, 10% Aqueous.** Dissolve 10 gm sodium tungstate ($Na_2WO_4 \cdot 2H_2O$—Mallinckrodt, Folin Special) in ammonia-free distilled water (1) and dilute to 100 ml. The solution must be neutral or slightly alkaline to phenolphthalein, not more than 0.4 ml being required to neutralize 10 ml 0.1 N hydrochloric acid. Preserve in resistant-glass bottle.
4. **Phenolphthalein, 0.25% Alcoholic.** Dissolve 250 mg phenolphthalein ($C_{20}H_{14}O_4$—Baker & Adamson, A.C.S.) in 100 ml absolute methyl alcohol (CH_3OH—Merck, A.C.S.).
5. **Sodium Hydroxide, Volumetric 0.1 N.** Prepare by diluting slightly more than the required amount of concentrated carbonate-free sodium hydroxide solution (see Appendix) and standardize against 0.1 N acid using phenolphthalein as indicator. Preserve in ceresin-lined bottle.
6. **Sodium Tetraborate, 2% Aqueous.** Dissolve 2 gm powdered sodium tetraborate ($Na_2B_4O_7 \cdot 10H_2O$—Baker & Adamson, A.C.S.) in ammonia-free distilled water (1) and dilute to 100 ml. Preserve in resistant-glass bottle.
7. **Sodium b - Naphthoquinone - 4 - Sulfonate, 0.5% Aqueous.** Dissolve 50 mg sodium b-naphthoquinone-4-sulfonate ($C_{10}H_5O_2(SO_3Na)$—Reagent Chemicals, Special) in 10 ml ammonia-free distilled water (1). Use within 20 minutes after preparation. Four ml of this solution, when diluted to 25 ml and treated with 1 ml 50% acetic acid and 1 ml 15% sodium thiosulfate should be almost completely decolorized in a few seconds.
8. **Acid-Formaldehyde Solution (Russell).** Mix equal volumes of 0.6 N hydrochloric acid (a) and 0.08 M formaldehyde (b).
   (a) Hydrochloric acid, volumetric 0.6 N; add 60 ml concentrated hydrochloric acid (HCl—Baker & Adamson, A.C.S.) to 1000 ml ammonia-free distilled water (1) and mix;

titrate against standard 1 N alkali using phenolphthalein as indicator; adjust accordingly and re-titrate—20 ml of 0.6 N acid require 12 ml of 1 N alkali.

(b) Formaldehyde, 0.08 M; dilute 6 ml 40% formaldehyde (HCHO—Baker & Adamson, Reagent) to 1000 ml with ammonia-free distilled water (1).

9. **Sodium Thiosulfate, 0.05 M.** Dissolve 1.241 gm crystalline sodium thiosulfate ($Na_2S_2O_3 \cdot 5H_2O$ —Mallinckrodt, Low-Sulfate Special) in ammonia-free distilled water (1) and dilute to 100 ml. This solution is best prepared just before use—there must be no white precipitate or odor of sulfide.

Technic

Note: For concentrations of amino acid nitrogen in excess of 20 mg per 100 ml, dilute one volume of the protein-free filtrate with three volumes of ammonia-free distilled water (1) before proceeding with Step 7 and multiply the values obtained from the calibration table by 4.

1. In a 30 ml Erlenmeyer flask place
   8 ml N/12 sulfuric acid (2).
2. Add 1 ml sample from an Ostwald-Folin pipette, thoroughly rinsing out the pipette by drawing in and expelling some of the mixture several times.
3. Mix the contents of the flask by gentle rotation until the fluid is uniformly black-brown.
4. Add 1 ml 10% sodium tungstate (3), drop by drop while constantly, but gently, rotating the flask.
5. Shake the flask vigorously until the mixture is chocolate colored and foam-free.
6. Filter through a dry 9 cm Whatman #4 paper into a dry test tube.
7. In a 25 ml graduated test tube (sample) place
   3 ml protein-free filtrate.
8. In another 25 ml graduated test tube (reagent blank) place
   3 ml ammonia-free distilled water (1).
9. To each tube add a small drop of 0.25% phenolphthalein (4) from a capillary-tipped dropper.
10. While gently agitating the tubes add 0.1 N sodium hydroxide (5) in small drops from another capillary-tipped dropper until one drop produces a faint but permanent pink color.
11. Dilute to 5 ml with ammonia-free distilled water (1).
12. Add 1 ml 2% sodium tetraborate (6) and mix—the color becomes fainter but is still a definite pink.
13. Add 2 ml freshly prepared 0.5% sodium b-naphthoquinone-4-sulfonate (7) and mix—the color is now a reddish-orange.
14. Place in boiling water for 10 minutes—the color changes to a deep brown.
15. Cool under running tap water for not more than 5 minutes—if too cold, a precipitate forms.
16. Dilute to 20 ml with ammonia-free distilled water (1) and mix.
17. Add 1.7 ml acid-formaldehyde solution (8) and mix.
18. Add 1.7 ml 0.05 M sodium thiosulfate (9) and mix—the excess brown color has been destroyed and a clear orange-red remains.
19. Dilute to 25 ml with ammonia-free distilled water (1) and mix.
20. Allow to stand at room temperature for 10 minutes.
21. Measure the transmittancy of the sample against the reagent blank set at 100% transmission at a wave length of 475 mμ and obtain the concentration of amino acid nitrogen from the calibration table.

CALIBRATION PROCEDURE

Special Apparatus

Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.

Graduated test tubes—10 ml in 0.1 ml; the 6 ml graduation must be accurately calibrated.

Graduated test tubes—25 ml in 0.5 ml; the 25 ml graduation must be accurately calibrated.

Capillary droppers delivering 40 drops per ml.

Reagents

1. **Water, Distilled, Ammonia-Free.** Same as for Test Procedure.

4. **Phenolphthalein, 0.25% Alcoholic.** Same as for Test Procedure.

5. **Sodium Hydroxide, Volumetric 0.1 N.** Same as for Test Procedure.

6. **Sodium Tetraborate, 2% Aqueous.** Same as for Test Procedure.

7. **Sodium b - Naphthoquinone - 4 - Sulfonate, 0.5% Aqueous.** Same as for Test Procedure.

8. **Acid-Formaldehyde Solution (Russell).** Same as for Test Procedure.

9. **Sodium Thiosulfate, 0.05 M.** Same as for Test Procedure.

10. **Hydrochloric Acid-Benzoate Solution.** In a 1000 ml volumetric flask place 2 gm sodium benzoate ($NaC_7H_5O_2$—Merck, U.S.P.); add approximately 800 ml ammonia-free distilled water (1) and shake until dissolved; add 70 ml 1 N hydrochloric acid (a); dilute to volume with ammonia-free distilled water. Filter, if necessary, before use.

    (a) Hydrochloric acid, volumetric 1 N; add 100 ml concentrated hydrochloric acid (HCl—Baker & Adamson, A.C.S.) to 1000 ml ammonia-free distilled water (1) and standardize against 1 N alkali using phenolphthalein as indicator.

11. **Amino Acid Standards**

    (a) Aminoacetic acid stock standard (1 ml contains 0.1 mg amino acid nitrogen). Dry aminoacetic acid ($C_2H_5O_2N$—Reagent

Chemicals, Ammonia-Free Special) over sulfuric acid in vacuo to constant weight; transfer 267.5 mg to a 500 ml volumetric flask; add approximately 400 ml hydrochloric acid-benzoate solution (10); shake until dissolved and dilute to volume with same. Stability indefinite.

(b) Proline stock standard (1 ml contains 0.1 mg amino acid nitrogen). Dry l(-)proline ($C_5H_9O_2N$—Merck) over sulfuric acid in vacuo to constant weight; transfer 187.8 mg to a 500 ml volumetric flask; add approximately 400 ml hydrochloric acid-benzoate solution (10); shake until dissolved and dilute to volume with same. Stability indefinite.

(c) Dilute standard (1 ml contains 0.024 mg amino acid nitrogen). In a 100 ml volumetric flask place 12 ml aminoacetic acid stock standard (11a) and 12 ml proline stock standard (11b); dilute to volume with ammonia-free distilled water (1); add a few drops of chloroform. Preserve in refrigerator. Stability about one week.

(d) Calibration standards. In a series of six accurately calibrated 10 ml test tubes place respectively 0, 1, 2, 3, 4 and 5 ml of the dilute standard (11c); add 6, 5, 4, 3, 2 and 1 ml ammonia-free distilled water (1) respectively, bringing the volume in each tube to 6 ml. These standards represent concentrations of amino acid nitrogen equivalent to 0, 4, 8, 12, 16 and 20 mg per 100 ml sample. Prepare just before use.

Technic

1. Transfer 3 ml of each calibration standard (11d) to a correspondingly marked 25 ml graduated test tube.
2. Proceed exactly as outlined in the Test Procedure, Steps 9–20 inclusive.
3. Set the zero concentration standard at 100% transmission at a wave length of **475 mμ** and record the transmittancy of the 4, 8, 12, 16 and 20 mg per cent standards. The transmittancy of the 20 mg standard should be approximately 23.5%.
4. Plot the observed values on semi-log graph paper—the points fall on a straight line which passes through the origin (Calibration Curve A-16).
5. Prepare a calibration table from the T-C values obtained from the plotted curve.

PROCEDURE FOR USE WITH LINEAR-SCALE PHOTOELECTRIC COLORIMETERS (SHEARD-SANFORD TYPE)

TEST PROCEDURE

Exactly as outlined; set the reagent blank at 100 and use a blue filter.

CALIBRATION PROCEDURE

Exactly as outlined—use a blue filter; plot the observed readings on semi-log graph paper—the points fall on a very slight curve passing through the origin.

PROCEDURE FOR USE WITH LOGARITHMIC-SCALE PHOTOELECTRIC COLORIMETERS (KLETT-SUMMERSON TYPE)

TEST PROCEDURE

Exactly as outlined; set the reagent blank at 0 and use a blue filter.

CALIBRATION PROCEDURE

Exactly as outlined—use a blue filter; plot the observed readings on linear graph paper—the points fall on a very slight curve passing through the origin.

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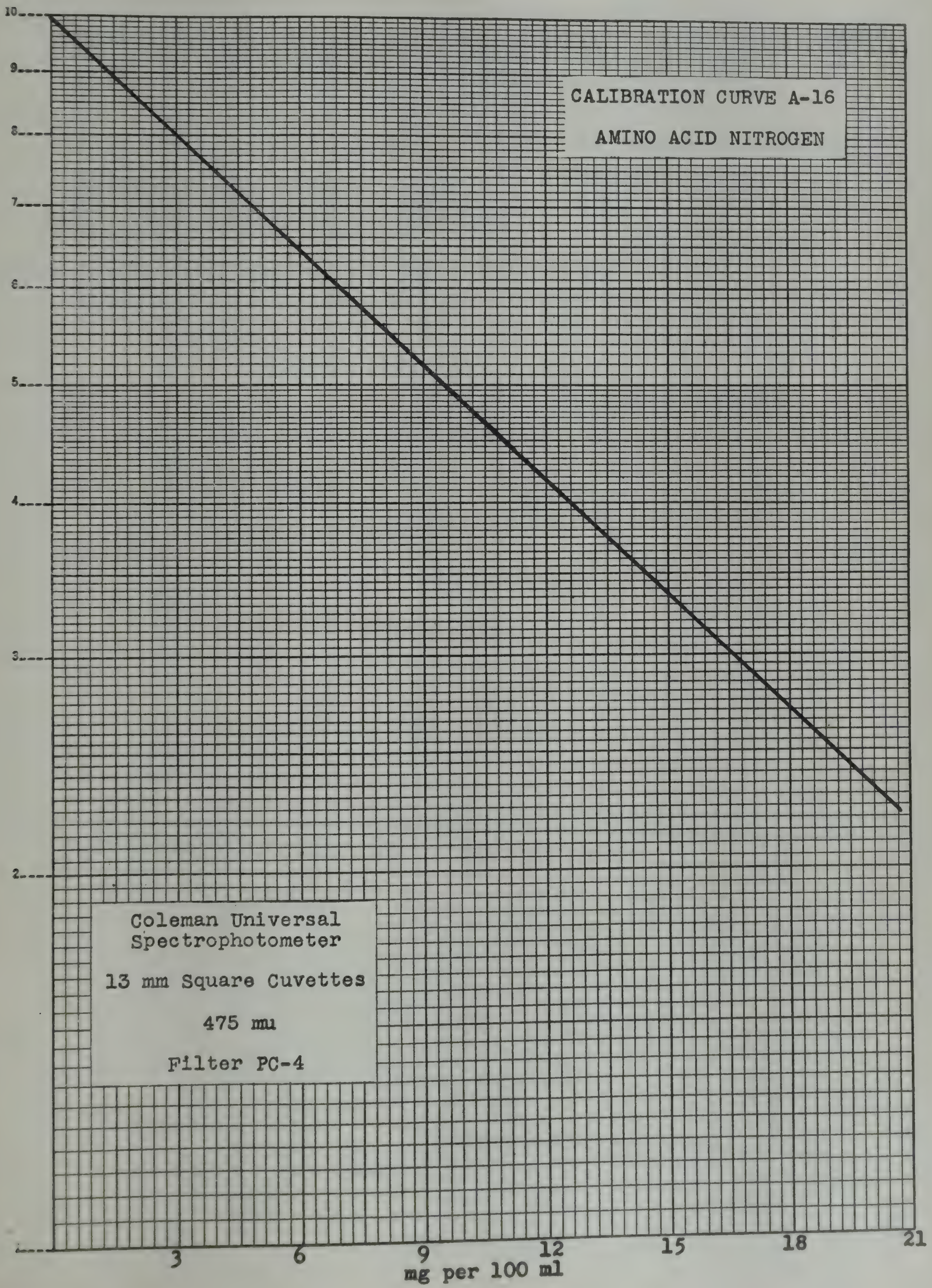
CALIBRATION CURVE A-16
AMINO ACID NITROGEN
Coleman Universal Spectrophotometer
13 mm Square Cuvettes
475 mμ
Filter PC-4
10
9
8
7
6
5
4
3
2
1
3
6
9
12
15
18
21
mg per 100 ml

Meter Reading	0	1	2	3	4	5	6	7	8	9
0										
10										
20										
30										
40										
50										
60										
70										
80										
90										

p-AMINOBENZOIC ACID, FREE

A spectrophotometric adaptation based on the methods and modifications of Marshall (1937), Marshall and Litchfield (1938), Bratton and Marshall (1939), Strauss, Lowell and Finland (1941) and Eckert (1943)

PRINCIPLE

The free amino group of p-aminobenzoic acid in a protein-free trichloracetic acid filtrate is diazotized with nitrous acid, the excess nitrite being destroyed by the addition of ammonium sulfamate. The diazotized p-aminobenzoic acid is coupled with N-(1-naphthyl)-ethylenediamine dihydrochloride to form an azo compound with a purplish-red color (Bratton-Marshall reaction).

DETERMINATION IN WHOLE BLOOD

SAMPLE

Blood withdrawn at any time of the day; after ingestion, p-aminobenzoic acid rapidly disappears from the blood stream and therefore the optimum time must be chosen for securing the blood sample. Sulfa compounds must not be administered when p-aminobenzoic acid determinations by this method are to be made.

TEST PROCEDURE

Special Apparatus

Ostwald-Folin pipettes accurately calibrated "to contain" 2 ml.

Graduated test tubes—25 ml in 0.5 ml; the 25 ml graduation must be accurately calibrated.

Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.

Reagents

1. **Trichloracetic Acid, 15% Aqueous.** Dilute 15 ml concentrated trichloracetic acid (a) to 100 ml with distilled water. Prepare fresh weekly. Preserve in glass-stoppered bottle.
    (a) Trichloracetic acid, concentrated; dissolve 100 gm crystalline trichloracetic acid (CCl_3-$COOH$—J. T. Baker, CP Analyzed) in distilled water and dilute to 100 ml. Preserve in glass-stoppered bottle. Stability indefinite.
2. **Trichloracetic Acid, 3% Aqueous.** Dilute 20 ml 15% trichloracetic acid (1) to 100 ml with distilled water. Preserve in refrigerator. Prepare fresh weekly.
3. **Sodium Nitrite, 0.2% Aqueous.** Dilute 2 ml 10% sodium nitrite (a) to 100 ml with distilled water. Prepare fresh daily and preserve in a brown glass bottle.
    (a) Sodium nitrite, 10% aqueous; dissolve 10 gm granular sodium nitrite ($NaNO_2$—Mallinckrodt, A.C.S.) in distilled water and dilute to 100 ml. Preserve in refrigerator.
4. **Ammonium Sulfamate, 2% Aqueous.** Dissolve 2 gm recrystallized ammonium sulfamate ($NH_2SO_3NH_4$—Reagent Chemicals, Special) in distilled water and dilute to 100 ml.
5. **N-(1-Naphthyl)-Ethylenediamine Dihydrochloride, 0.1% Aqueous (Bratton-Marshall).** Dissolve 100 mg N-(1-naphthyl)-ethylenediamine dihydrochloride ($C_{10}H_7HNCH_2CH_2NH_2 \cdot 2HCl$—Eastman #4835) in 100 ml distilled water. Preserve in a brown bottle in refrigerator. Stability about one month. Discard when a brownish color appears.

Technic

Note: If total p-aminobenzoic acid (Method A-21) is to be determined on the same sample, reserve a portion of the trichloracetic acid filtrate in Step 7.

1. In a 50 ml Erlenmeyer flask place 30 ml distilled water.
2. Add 2 ml oxalated whole blood from an Ostwald-Folin pipette, thoroughly rinsing out the pipette by drawing in and expelling some of the mixture several times.
3. Allow to stand for 5 minutes until laking is complete.
4. Slowly add 8 ml 15% trichloracetic acid (1), drop by drop, with constant agitation of the flask.
5. Shake vigorously until foam-free.
6. Allow to stand for 15 minutes.
7. Filter through a dry 12.5 cm Whatman #40 paper into a dry test tube—RESERVE PORTION FOR TOTAL p-AMINOBENZOIC ACID ANALYSIS (Method A-21).
8. In a 25 ml graduated test tube (sample) place
    5 ml protein-free filtrate.
9. In another 25 ml graduated test tube (reagent blank) place
    5 ml 3% trichloracetic acid (2).
10. To each tube add 1 ml distilled water.
11. Add 0.5 ml 0.2% sodium nitrite (3) and mix.
12. Allow to stand for 15 minutes.
13. Add 0.5 ml 2% ammonium sulfamate (4) and mix well.
14. Allow to stand for 3 minutes.
15. Add 2.5 ml 0.1% N-(1-naphthyl)-ethylenediamine dihydrochloride (5) and mix.
16. Allow to stand for 60 minutes.
17. Dilute to 25 ml with distilled water.

18. Measure the transmittancy of the sample against the reagent blank set at 100% transmission at a wave length of 540 mμ and obtain the concentration of free p-aminobenzoic acid from the calibration table.

CALIBRATION PROCEDURE

Special Apparatus

Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.

Graduated test tubes—25 ml in 0.5 ml; the 25 ml graduation must be accurately calibrated.

Reagents

2. **Trichloracetic Acid, 3% Aqueous.** Same as for Test Procedure.
3. **Sodium Nitrite, 0.2% Aqueous.** Same as for Test Procedure.
4. **Ammonium Sulfamate, 2% Aqueous.** Same as for Test Procedure.
5. **N-(1-Naphthyl)-Ethylenediamine Dihydrochloride, 0.1% Aqueous (Bratton-Marshall).** Same as for Test Procedure.
6. **p-Aminobenzoic Acid Standards**
   (a) p-Aminobenzoic acid stock standard (1 ml contains 1 mg p-aminobenzoic acid). In a 100 ml volumetric flask place 100 mg recrystallized p-aminobenzoic acid ($NH_2C_6H_4COOH$—Reagent Chemicals, Special); add approximately 50 ml 3% trichloracetic acid (2) and shake until dissolved; dilute to volume with same.
   (b) Dilute standard (1 ml contains 0.01 mg p-aminobenzoic acid). In a 100 ml volumetric flask place 1 ml stock standard (6a) and dilute to volume with 3% trichloracetic acid (2). Prepare just before use.
   (c) Calibration standards. In a series of seven accurately calibrated 25 ml graduated test tubes place respectively 0, 0.5, 1, 2, 3, 4 and 5 ml of the dilute standard (6b); add 5, 4.5, 4, 3, 2, 1 and 0 ml 3% trichloracetic acid (2) respectively, bringing the volume in each tube to 5 ml. These standards represent concentrations of p-aminobenzoic acid equivalent to 0, 2, 4, 8, 12, 16 and 20 mg per 100 ml sample. Prepare just before use.

Technic

1. Treat each calibration standard (6c) exactly as outlined in the Test Procedure, Steps 10–17 inclusive.
2. Set the zero concentration standard at 100% transmission at a wave length of 540 mμ and record the transmittancy of the 2, 4, 8, 12, 16 and 20 mg per cent standards. The transmittancy of the 20 mg standard should be approximately 17%.
3. Plot the observed values on semi-log graph paper—the points fall on a very slight curve which passes through the origin (Calibration Curve A-20).
4. Prepare a calibration table from the T-C values obtained from the plotted curve.

Method A-20b

DETERMINATION IN URINE

SAMPLE

Aliquot of a 24-hour specimen.

TEST PROCEDURE

Special Apparatus

As listed under the Test Procedure for blood.

Reagents

1. **Trichloracetic Acid, 15% Aqueous.** Same as for blood.
2. **Trichloracetic Acid, 3% Aqueous.** Same as for blood.
3. **Sodium Nitrite, 0.2% Aqueous.** Same as for blood.
4. **Ammonium Sulfamate, 2% Aqueous.** Same as for blood.
5. **N-(1-Naphthyl)-Ethylenediamine Dihydrochloride, 0.1% Aqueous (Bratton-Marshall).** Same as for blood.

Technic

Note: For concentrations of p-aminobenzoic acid in excess of 20 mg per 100 ml, dilute one volume of filtered urine with an equal volume of distilled water before proceeding with Step 3 and multiply the values obtained from the calibration table for blood by 2.

1. Filter a small portion of thoroughly mixed urine.
2. In a 50 ml Erlenmeyer flask place 30 ml distilled water.
3. Add 2 ml filtered urine from an Ostwald-Folin pipette, thoroughly rinsing out the pipette by drawing in and expelling some of the mixture several times.

4. Proceed exactly as outlined in the Test Procedure for blood, Steps 4–17 inclusive.
5. Measure the transmittancy of the sample against the reagent blank set at 100% transmission at a wave length of **540** mμ and obtain the concentration of free p-aminobenzoic acid from the calibration table for blood.

PROCEDURE FOR USE WITH LINEAR-SCALE PHOTOELECTRIC COLORIMETERS (SHEARD-SANFORD TYPE)

TEST PROCEDURE

Exactly as outlined; set the reagent blank at 100 and use a green filter.

CALIBRATION PROCEDURE

Exactly as outlined—use a green filter; plot the observed readings on semi-log graph paper—the points fall on a very slight curve passing through the origin.

PROCEDURE FOR USE WITH LOGARITHMIC-SCALE PHOTOELECTRIC COLORIMETERS (KLETT-SUMMERSON TYPE)

TEST PROCEDURE

Exactly as outlined; set the reagent blank at 0 and use a green filter.

CALIBRATION PROCEDURE

Exactly as outlined—use a green filter; plot the observed readings on linear graph paper—the points fall on a very slight curve passing through the origin.

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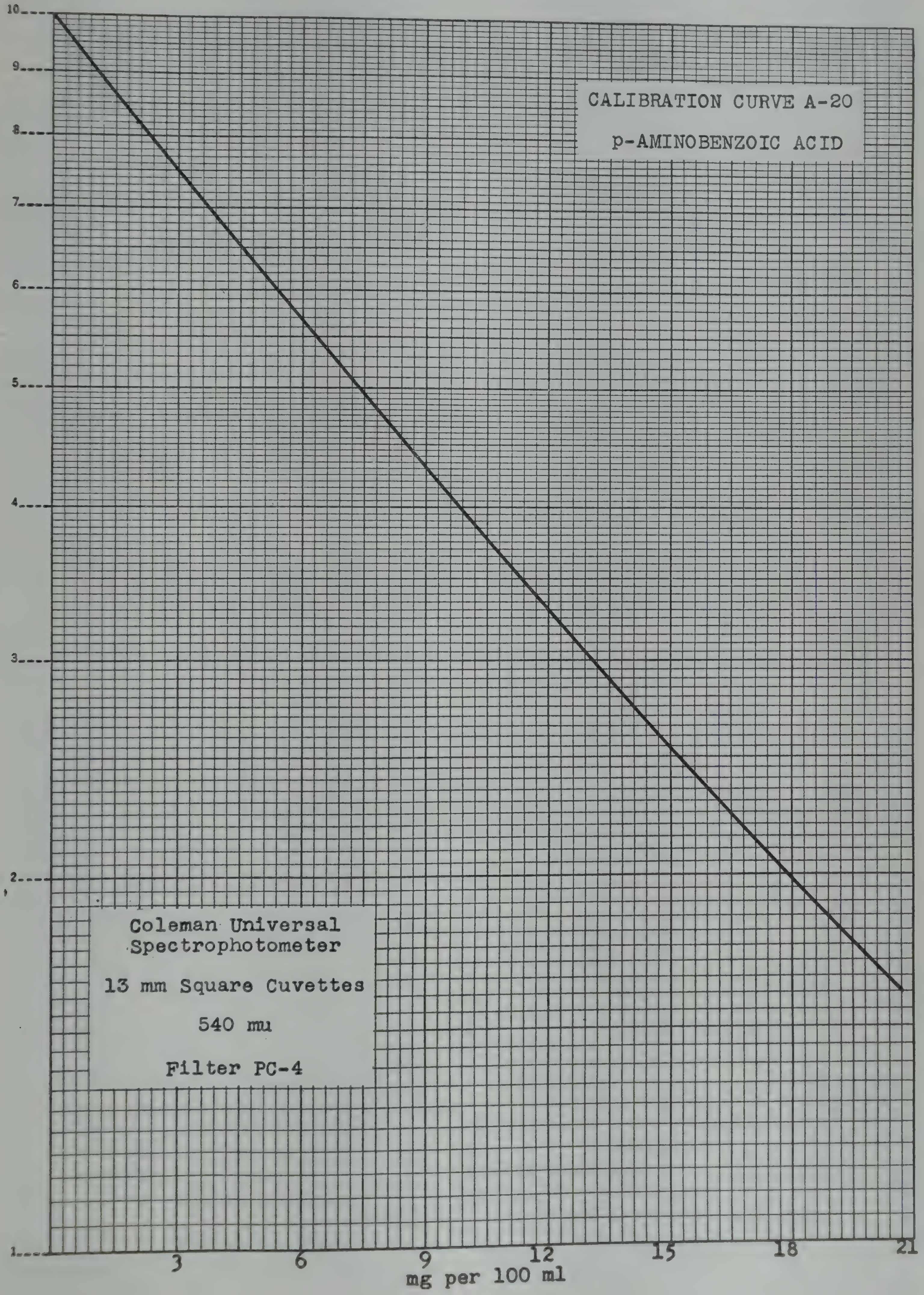
CALIBRATION CURVE A-20
p-AMINOBENZOIC ACID
Coleman Universal Spectrophotometer
13 mm Square Cuvettes
540 mμ
Filter PC-4
10
9
8
7
6
5
4
3
2
1
3
6
9
12
15
18
21
mg per 100 ml

Meter Reading	0	1	2	3	4	5	6	7	8	9
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70										
80										
90										

p-AMINOBENZOIC ACID, TOTAL

A spectrophotometric adaptation based on the methods and modifications of Marshall (1937), Marshall and Litchfield (1938), Bratton and Marshall (1939), Strauss, Lowell and Finland (1941) and Eckert (1943)

PRINCIPLE

The amino group of the free p-aminobenzoic acid and the amino group split off from the conjugated form by means of acid hydrolysis are determined together according to the analytical principles described for free p-aminobenzoic acid (Method A-20).

DETERMINATION IN WHOLE BLOOD

SAMPLE

See remarks under free p-aminobenzoic acid (Method A-20).

TEST PROCEDURE

Special Apparatus

As listed under the Test Procedure for free p-aminobenzoic acid.

Reagents

1. **Trichloracetic Acid, 15% Aqueous.** Same as for free p-aminobenzoic acid.
2. **Trichloracetic Acid, 3% Aqueous.** Same as for free p-aminobenzoic acid.
3. **Sodium Nitrite, 0.2% Aqueous.** Same as for free p-aminobenzoic acid.
4. **Ammonium Sulfamate, 2% Aqueous.** Same as for free p-aminobenzoic acid.
5. **N-(1-Naphthyl)-Ethylenediamine Dihydrochloride, 0.1% Aqueous (Bratton-Marshall).** Same as for free p-aminobenzoic acid.
7. **Hydrochloric Acid, Volumetric 4 N.** Add 400 ml concentrated hydrochloric acid (HCl—Baker & Adamson, A.C.S.) to 600 ml distilled water and mix; allow to cool; dilute 25 ml of this approximately 4 N solution to 100 ml; titrate against standard 1 N alkali using phenolphthalein as indicator; adjust accordingly and re-titrate—20 ml of a 1:4 dilution of 4 N acid require 20 ml of 1 N alkali.

Technic

Note: If the analysis is to be carried out on an aliquot of the trichloracetic acid filtrate as reserved in the determination of free p-aminobenzoic acid (Method A-20, Step 7), omit the following Steps 1–7 inclusive.

1. In a 50 ml Erlenmeyer flask place 30 ml distilled water.
2. Add 2 ml oxalated whole blood from an Ostwald-Folin pipette, thoroughly rinsing out the pipette by drawing in and expelling some of the mixture several times.
3. Allow to stand for 5 minutes until laking is complete.
4. Slowly add 8 ml 15% trichloracetic acid (1), drop by drop, with constant agitation of the flask.
5. Shake vigorously until foam-free.
6. Allow to stand for 15 minutes.
7. Filter through a dry 12.5 cm Whatman #40 paper into a dry test tube.
8. In a 25 ml graduated test tube (sample) place
   5 ml protein-free filtrate.
9. Add 0.3 ml 4 N hydrochloric acid (7) and mix.
10. Place in boiling water for 60 minutes.
11. Cool to room temperature under running tap water.
12. Add sufficient distilled water to bring the volume to the original 5 ml level.
13. In another 25 ml graduated test tube (reagent blank) place
   5 ml 3% trichloracetic acid (2)
   0.3 ml 4 N hydrochloric acid (7) and mix.
14. To each tube add 1 ml distilled water.
15. Add 0.5 ml 0.2% sodium nitrite (3) and mix.
16. Allow to stand for 15 minutes.
17. Add 0.5 ml 2% ammonium sulfamate (4) and mix well.
18. Allow to stand for 3 minutes.
19. Add 2.5 ml 0.1% N-(1-naphthyl)-ethylenediamine dihydrochloride (5) and mix.
20. Allow to stand for 60 minutes.
21. Dilute to 25 ml with distilled water.
22. Measure the transmittancy of the sample against the reagent blank set at 100% transmission at a wave length of 540 mμ and obtain the concentration of total p-aminobenzoic acid directly from the calibration table for free p-aminobenzoic acid (Method A-20).

CALIBRATION PROCEDURE

Values are read directly from the calibration table for free p-aminobenzoic acid (Method A-20).

Method A-21b

DETERMINATION IN URINE

SAMPLE

See remarks under free p-aminobenzoic acid (Method A-20).

TEST PROCEDURE

Special Apparatus

As listed under the Test Procedure for free p-aminobenzoic acid.

Reagents

1. **Trichloracetic Acid, 15% Aqueous.** Same as for free p-aminobenzoic acid.
2. **Trichloracetic Acid, 3% Aqueous.** Same as for free p-aminobenzoic acid.
3. **Sodium Nitrite, 0.2% Aqueous.** Same as for free p-aminobenzoic acid.
4. **Ammonium Sulfamate, 2% Aqueous.** Same as for free p-aminobenzoic acid.
5. **N-(1-Naphthyl)-Ethylenediamine Dihydrochloride, 0.1% Aqueous (Bratton-Marshall).** Same as for free p-aminobenzoic acid.
7. **Hydrochloric Acid, Volumetric 4 N.** Same as for blood.

Technic

Note: For concentrations of total p-aminobenzoic acid in excess of 20 mg per 100 ml, dilute the protein-free filtrate in Step 7 with an amount of 3% trichloracetic acid (2) so as to bring the concentration below the 20 mg per 100 ml level and multiply the values obtained from the calibration table for free p-aminobenzoic acid (Method A-20) by the appropriate dilution factor.

1. Filter a small portion of thoroughly mixed urine.
2. In a 50 ml Erlenmeyer flask place 30 ml distilled water.
3. Add 2 ml filtered urine from an Ostwald-Folin pipette, thoroughly rinsing out the pipette by drawing in and expelling some of the mixture several times.
4. Slowly add 8 ml 15% trichloracetic acid (1), drop by drop, with constant agitation of the flask.
5. Shake vigorously until foam-free.
6. Allow to stand for 15 minutes.
7. Filter through a dry 12.5 cm Whatman #40 paper into a dry test tube.
8. In a 25 ml graduated test tube (sample) place
   5 ml protein-free filtrate.
9. Add 5 ml 4 N hydrochloric acid (7) and mix.
10. Place in boiling water for 60 minutes.
11. Cool to room temperature under running tap water.
12. Add sufficient distilled water to bring the volume to the original 10 ml level.
13. In another 25 ml graduated test tube (reagent blank) place
    5 ml 3% trichloracetic acid (2)
    5 ml 4 N hydrochloric acid (7) and mix.
14. Proceed exactly as outlined in the Test Procedure for blood, Steps 14–21, inclusive.
15. Measure the transmittancy of the sample against the reagent blank set at 100% transmission at a wave length of **540** mμ and obtain the concentration of total p-aminobenzoic acid directly from the calibration table for free p-aminobenzoic acid in blood (Method A-20).

◆

PROCEDURE FOR USE WITH LINEAR-SCALE PHOTOELECTRIC COLORIMETERS (SHEARD-SANFORD TYPE)

TEST PROCEDURE

Exactly as outlined; set the reagent blank at 100 and use a green filter.

CALIBRATION PROCEDURE

Need not be performed; values obtained directly from calibration table for free p-aminobenzoic acid.

◆

PROCEDURE FOR USE WITH LOGARITHMIC-SCALE PHOTOELECTRIC COLORIMETERS (KLETT-SUMMERSON TYPE)

TEST PROCEDURE

Exactly as outlined; set the reagent blank at 0 and use a green filter.

CALIBRATION PROCEDURE

Need not be performed; values obtained directly from calibration table for free p-aminobenzoic acid.

BIBLIOGRAPHY

Same as listed under free p-aminobenzoic acid (Method A-20).

p-AMINOBENZOIC ACID, FREE

A spectrophotometric adaptation based on the method of Kirch and Bergeim (1943)

PRINCIPLE

Reducing substances in acidified urine are oxidized by means of iodine, the color of which is then destroyed by sodium bisulfite. The p-aminobenzoic acid is diazotized by addition of sodium nitrite and coupled with thiamine chloride to produce a red color (Kirch-Bergeim reaction), which is extracted with iso-propyl ether. Sulfonamides also produce color by this reaction but these colors are not extractable.

DETERMINATION IN URINE

SAMPLE

Aliquot of a 24-hour specimen.

TEST PROCEDURE

Special Apparatus

Separatory funnels, 60 ml, globe shaped.
Capillary droppers delivering 40 drops per ml.
Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.

Reagents

1. **Acetic Acid, 35% Aqueous.** Dilute 35 ml glacial acetic acid (CH_3COOH—Baker & Adamson, A.C.S.) to 100 ml with distilled water.
2. **Iodine, Volumetric 0.1 N.** In a 1000 ml volumetric flask place 13 gm resublimed iodine crystals (I—Baker & Adamson, A.C.S.) and 30 gm potassium iodide (KI—J. T. Baker, Low-Iodate, Special); add approximately 100 ml distilled water and shake until the iodine is completely dissolved; dilute to volume with distilled water and mix; titrate against standard 0.1 N sodium thiosulfate using starch as indicator; adjust accordingly and re-titrate. Restandardize at frequent intervals.
3. **Sodium Bisulfite, 1% Aqueous.** Dissolve 1 gm sodium bisulfite ($NaHSO_3$—Merck, Reagent) in distilled water and dilute to 100 ml. Prepare fresh weekly.
4. **Thiamine Reagent (Kirch-Bergeim).** Mix equal volumes of 0.2% thiamine chloride (a) and 2% sodium nitrite (b). Prepare the amount needed just before use.
   (a) Thiamine chloride, 0.2% aqueous; dissolve 200 mg thiamine hydrochloride ($C_{12}H_{17}ON_4SCl \cdot HCl$—Merck, U.S.P.) in distilled water and dilute to 100 ml. Preserve in refrigerator.
   (b) Sodium nitrite, 2% aqueous; dissolve 2 gm sodium nitrite ($NaNO_2$—Mallinckrodt, A.C.S.) in distilled water and dilute to 100 ml. Preserve in refrigerator. Stability approximately one month.
5. **Sodium Hydroxide, Volumetric 1 N.** Prepare by diluting slightly more than the required amount of concentrated carbonate-free sodium hydroxide solution (see Appendix) and standardize against 1 N acid using phenolphthalein as indicator. Preserve in ceresin-lined bottle.
6. **iso-Propyl Ether, Alcohol-Free** ($[(CH_3)_2CH]_2O$—Eastman #1193).

Technic

Note: For concentrations of p-aminobenzoic acid in excess of 15 mg per 100 ml, dilute the filtered urine with an equal volume of distilled water before carrying out Step 2 and multiply the values obtained from the calibration table by 2. For very low concentrations use more urine and correspondingly less water in Step 2 and divide the values obtained from the calibration table by the appropriate factor. (A urine sample of 5 ml may sometimes be necessary.)

1. Filter a small portion of thoroughly mixed urine.
2. In a 60 ml separatory funnel (sample) place
   1 ml filtered urine
   19 ml distilled water.
3. In another 60 ml separatory funnel (reagent blank) place
   20 ml distilled water.
4. To each funnel add 0.3 ml 35% acetic acid (1) and mix.
5. With gentle agitation, add 0.1 N iodine (2) from a capillary-tipped dropper until a permanent brown color appears.
6. Add 1 drop 1% sodium bisulfite (3) for every drop of iodine required in Step 5 and mix—the brown color disappears.
7. Add 5 ml thiamine reagent (4); mix and allow to stand for 15 minutes.
8. Add 4 ml 1 N sodium hydroxide (5); mix and allow to stand for 15 minutes—depending upon the concentration of p-aminobenzoic acid the color varies from a pale yellow to a deep amber.
9. Add 5 ml iso-propyl ether (6)
   0.5 ml 35% acetic acid (1).
10. Shake thoroughly for 2 minutes.
11. Allow to stand until the phases separate—the ether layer in the reagent blank is colorless; in the sample it varies from pink to red in color.
12. Draw off all of the aqueous layer and a few drops of the ether layer and discard.
13. Transfer the ether layers to dry cuvettes through dry 5 cm filter papers—this removes traces of moisture from the ether.

14. Measure the transmittancy of the sample against the reagent blank set at 100% transmission at a wave length of 500 mμ and obtain the concentration of free p-aminobenzoic acid from the calibration table.

CALIBRATION PROCEDURE

Special Apparatus

Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.
Separatory funnels, 60 ml, globe shaped.
Capillary droppers delivering 40 drops per ml.

Reagents

1. **Acetic Acid, 35% Aqueous.** Same as for Test Procedure.
2. **Iodine, Volumetric 0.1 N.** Same as for Test Procedure.
3. **Sodium Bisulfite, 1% Aqueous.** Same as for Test Procedure.
4. **Thiamine Reagent (Kirch-Bergeim).** Same as for Test Procedure.
5. **Sodium Hydroxide, Volumetric 1 N.** Same as for Test Procedure.
6. **iso-Propyl Ether, Alcohol-Free.** Same as for Test Procedure.
7. **p-Aminobenzoic Acid Standards**
    - (a) p-Aminobenzoic acid stock standard (1 ml contains 1 mg p-aminobenzoic acid). In a 100 ml volumetric flask place 100 mg recrystallized p-aminobenzoic acid ($NH_2C_6H_4COOH$—Reagent Chemicals, Special); dilute to volume with distilled water and shake thoroughly until completely dissolved—solution is rather slow; adjust volume, if necessary, with distilled water.
    - (b) Dilute standard (1 ml contains 0.03 mg p-aminobenzoic acid). In a 100 ml volumetric flask place 3 ml stock standard (7a) and dilute to volume with distilled water. Prepare just before use.
    - (c) Calibration standards. In a series of six 60 ml separatory funnels place respectively 0, 1, 2, 3, 4 and 5 ml of the dilute standard (7b); add 20, 19, 18, 17, 16 and 15 ml distilled water respectively, bringing the volume in each funnel to 20 ml. These standards represent concentrations of p-aminobenzoic acid equivalent to 0, 3, 6, 9, 12 and 15 mg per 100 ml sample. Prepare just before use and proceed as follows.

Technic

1. Treat each calibration standard (7c) exactly as outlined in the Test Procedure, Steps 4–13 inclusive.
2. Set the zero concentration standard at 100% transmission at a wave length of 500 mμ and record the transmittancy of the 3, 6, 9, 12 and 15 mg per cent standards. The transmittancy of the 15 mg standard should be approximately 29%.
3. Plot the observed values on semi-log graph paper—the points fall on a straight line which passes through the concentration axis slightly to the right of the origin (Calibration Curve A-22).
4. Prepare a calibration table from the T-C values obtained from the plotted curve.

PROCEDURE FOR USE WITH LINEAR-SCALE PHOTOELECTRIC COLORIMETERS (SHEARD-SANFORD TYPE)

TEST PROCEDURE

Exactly as outlined; set the reagent blank at 100 and use a green filter.

CALIBRATION PROCEDURE

Exactly as outlined—use a green filter; plot the observed readings on semi-log graph paper—the points fall on a straight line which passes through the concentration axis slightly to the right of the origin.

PROCEDURE FOR USE WITH LOGARITHMIC-SCALE PHOTOELECTRIC COLORIMETERS (KLETT-SUMMERSON TYPE)

TEST PROCEDURE

Exactly as outlined; set the reagent blank at 0 and use a green filter.

CALIBRATION PROCEDURE

Exactly as outlined—use a green filter; plot the observed readings on linear graph paper—the points fall on a straight line which passes through the concentration axis slightly to the right of the origin.

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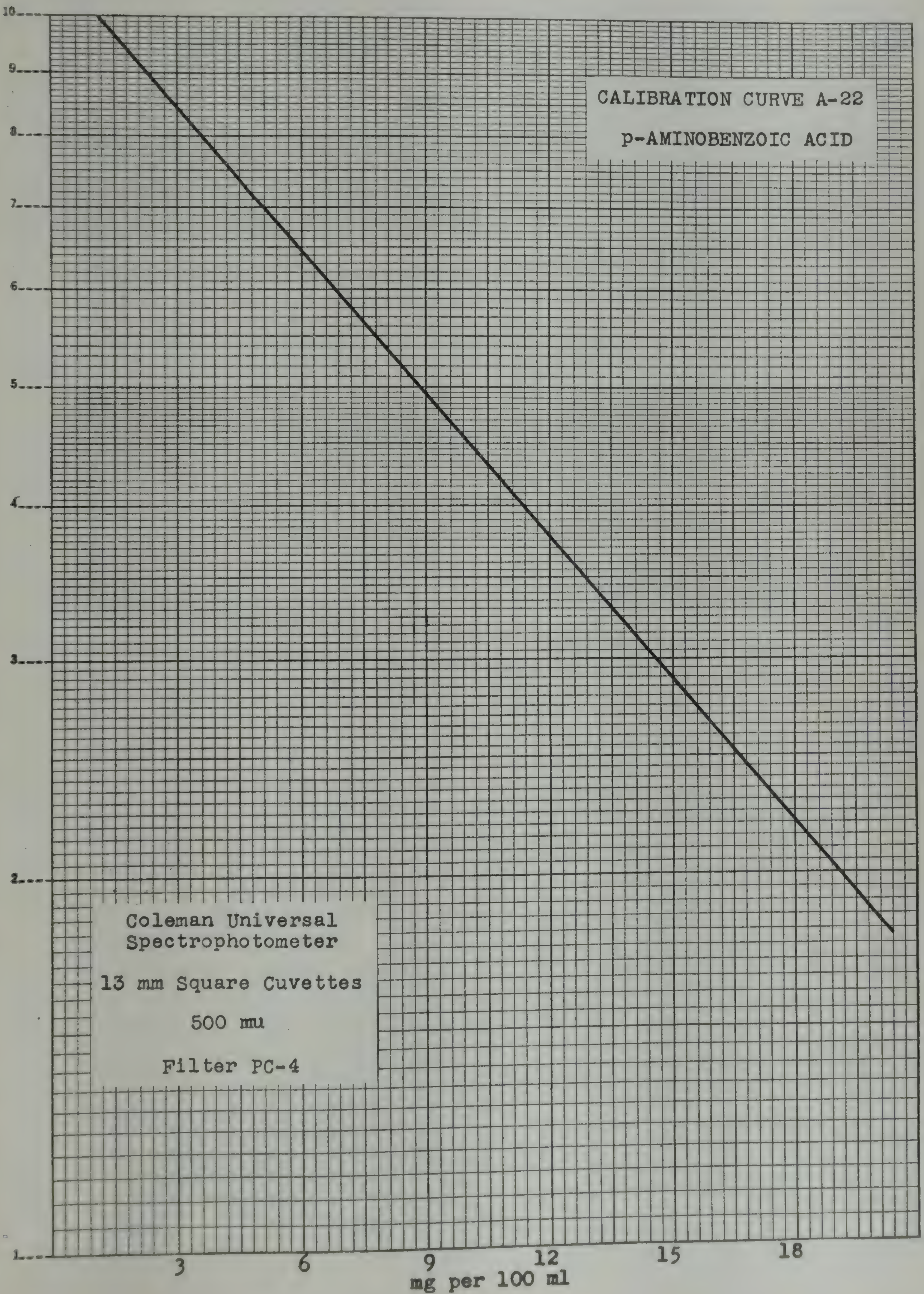
CALIBRATION CURVE A-22
p-AMINOBENZOIC ACID
Coleman Universal Spectrophotometer
13 mm Square Cuvettes
500 mu
Filter PC-4
10
9
8
7
6
5
4
3
2
1
3
6
9
12
15
18
mg per 100 ml

Meter Reading	0	1	2	3	4	5	6	7	8	9
0										
10										
20										
30										
40										
50										
60										
70										
80										
90										

p-AMINOBENZOIC ACID, TOTAL

A spectrophotometric adaptation based on the method of Kirch and Bergeim (1943)

PRINCIPLE

The amino group of the free p-aminobenzoic acid and the amino group split off from the conjugated form by means of acid hydrolysis are determined together according to the analytical principles described for free p-aminobenzoic acid (Method A-22).

DETERMINATION IN URINE

SAMPLE

See remarks under free p-aminobenzoic acid (Method A-22).

TEST PROCEDURE

Special Apparatus

Graduated test tubes—25 ml in 0.5 ml.
Separatory funnels, 60 ml, globe shaped.
Capillary droppers delivering 40 drops per ml.
Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.

Reagents

1. **Acetic Acid, 35% Aqueous.** Same as for free p-aminobenzoic acid.
2. **Iodine, Volumetric 0.1 N.** Same as for free p-aminobenzoic acid.
3. **Sodium Bisulfite, 1% Aqueous.** Same as for free p-aminobenzoic acid.
4. **Thiamine Reagent (Kirch-Bergeim).** Same as for free p-aminobenzoic acid.
5. **Sodium Hydroxide, Volumetric 1 N.** Same as for free p-aminobenzoic acid.
6. **iso-Propyl Ether, Alcohol-Free.** Same as for free p-aminobenzoic acid.
8. **Hydrochloric Acid, Volumetric 4 N.** Add 400 ml concentrated hydrochloric acid (HCl—Baker & Adamson, A.C.S.) to 600 ml distilled water and mix; allow to cool; dilute 25 ml of this approximately 4 N solution to 100 ml; titrate against standard 1 N alkali using phenolphthalein as indicator; adjust accordingly and re-titrate—20 ml of a 1:4 dilution of 4 N acid require 20 ml of 1 N alkali.

Technic

Note: See remarks under free p-aminobenzoic acid (Method A-22).

1. Filter a small portion of thoroughly mixed urine.
2. In a 25 ml graduated test tube place
   1 ml filtered urine
   8 ml distilled water
   1 ml 4 N hydrochloric acid (8).
3. Heat in boiling water for 60 minutes.
4. Cool to room temperature under running tap water.
5. Dilute to 15 ml with distilled water and transfer to a 60 ml separatory funnel (sample), rinse out the tube with a 5 ml portion of distilled water and transfer to the funnel.
6. In another 60 ml separatory funnel (reagent blank) place
   19 ml distilled water
   1 ml 4 N hydrochloric acid (8).
7. To each funnel add 5 ml iso-propyl ether (6), shake thoroughly, allow the phases to separate and transfer the aqueous layers with a few drops of the ether layer to another set of correspondingly marked funnels—the ether layer is discarded.
8. Repeat Step 7.
9. To each funnel add 0.3 ml 35% acetic acid (1) and mix.
10. With gentle agitation, add 0.1 N iodine (2) from a capillary-tipped dropper until a permanent brown color appears.
11. Add 1 drop 1% sodium bisulfite (3) for every drop of iodine required in Step 10 and mix.
12. Add 5 ml thiamine reagent (4); mix and allow to stand for 15 minutes.
13. Add 6 ml 1 N sodium hydroxide (5); mix and allow to stand for 15 minutes.
14. Add 5 ml iso-propyl ether (6)
    0.5 ml 35% acetic acid (1).
15. Shake thoroughly for 2 minutes.
16. Allow to stand until the phases separate.
17. Draw off all of the aqueous layer and a few drops of the ether layer and discard.
18. Transfer the ether layers to dry cuvettes through dry 5 cm filter papers.
19. Measure the transmittancy of the sample against the reagent blank set at 100% transmission at a wave length of 500 mμ and obtain the concentration of total p-aminobenzoic acid from the calibration table for free p-aminobenzoic acid (Method A-22).

CALIBRATION PROCEDURE

Values are read directly from the calibration table for free p-aminobenzoic acid (Method A-22).

PROCEDURE FOR USE WITH LINEAR-SCALE PHOTOELECTRIC COLORIMETERS (SHEARD-SANFORD TYPE)

TEST PROCEDURE

Exactly as outlined set the reagent blank at 100 and use a green filter.

CALIBRATION PROCEDURE

Need not be performed; values obtained directly from calibration table for free p-aminobenzoic acid.

PROCEDURE FOR USE WITH LOGARITHMIC-SCALE PHOTOELECTRIC COLORIMETERS (KLETT-SUMMERSON TYPE)

TEST PROCEDURE

Exactly as outlined; set the reagent blank at 0 and use a green filter.

CALIBRATION PROCEDURE

Need not be performed; values obtained directly from calibration table for free p-aminobenzoic acid.

BIBLIOGRAPHY

Same as listed under free p-aminobenzoic acid (Method A-22).

NOTES

p-AMINOBENZOIC ACID, CONJUGATED

The p-aminobenzoic acid in the conjugated form is the difference between the amounts of total and free p-aminobenzoic acid.

1. Determine the concentration of total p-aminobenzoic acid by either Method A-21 or Method A-23.
2. Determine the concentration of free p-aminobenzoic acid by either Method A-20 or Method A-22.
3. Calculation—

   Concentration of total p-aminobenzoic acid in mg per 100 ml − Concentration of free p-aminobenzoic acid in mg per 100 ml = Conjugated p-aminobenzoic acid in mg per 100 ml.

Note: When determining conjugated p-aminobenzoic acid, both the total and free determinations must be made by similar procedures, i.e., a combination of Methods A-20 and A-21 or Methods A-22 and A-23.

NOTES

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NOTES

p-AMINOHIPPURIC ACID, FREE

A spectrophotometric adaptation based on the methods and modifications of Bratton and Marshall (1939), Smith, Finkelstein, Aliminosa, Crawford and Graber (1945) and Hamburger, Ryckewaert, Duizend and Argant (1948)

PRINCIPLE

The free amino group of p-aminohippuric acid in a protein-free filtrate is diazotized with nitrous acid, the excess nitrite being destroyed by the addition of ammonium sulfamate. The diazotized p-aminohippuric acid is coupled with N-(1-naphthyl)-ethylenediamine dihydrochloride to form an azo compound with a purplish-red color (Bratton-Marshall reaction).

DETERMINATION IN PLASMA OR SERUM

SAMPLE

Plasma or serum from blood withdrawn during post-absorptive state. Separate plasma or serum from the red cells as soon as possible and store in refrigerator until use. Sulfa compounds must not be administered when p-aminohippuric acid determinations by this method are to be made.

TEST PROCEDURE

Special Apparatus

Ostwald-Folin pipettes accurately calibrated "to contain" 1 ml.

Graduated test tubes—10 ml in 0.1 ml; the 10 ml graduation must be accurately calibrated.

Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.

Reagents

1. **Acid Cadmium Sulfate Solution (Smith et al.).** In a 1000 ml volumetric flask place 34.667 gm crystalline cadmium sulfate ($3CdSO_4 \cdot 8H_2O$—Baker & Adamson, A.C.S.) or 28.24 gm anhydrous cadmium sulfate ($CdSO_4$—J. T. Baker, A.C.S.) and 169.3 ml 1 N sulfuric acid (a); add approximately 800 ml distilled water and shake until dissolved; dilute to volume with distilled water and filter. Note: If crystals of cadmium sulfate are used they must be clear and show no efflorescence.
   (a) Sulfuric acid, volumetric 1 N; slowly add 30 ml concentrated sulfuric acid (H_2SO_4—Grasselli, A.C.S.) to 1000 ml distilled water and mix; allow to cool; standardize against 1 N alkali using phenolphthalein as indicator.
2. **Sodium Hydroxide, Volumetric 1.1 N.** Prepare by diluting slightly more than the required amount of concentrated carbonate-free sodium hydroxide solution (see Appendix) and titrate against standard 1 N acid using phenolphthalein as indicator; adjust accordingly and re-titrate—20 ml of 1 N acid require 18.2 ml of 1.1 N alkali. Preserve in ceresin-lined bottle.
3. **Hydrochloric Acid, Volumetric 1.2 N.** Slowly add 110 ml concentrated hydrochloric acid (HCl—Baker & Adamson, A.C.S.) to 1000 ml distilled water and mix; allow to cool; titrate against standard 1 N alkali using phenolphthalein as indicator; adjust accordingly and re-titrate—20 ml of 1.2 N acid require 24 ml of 1 N alkali.
4. **Sodium Nitrite, 0.1% Aqueous.** Dilute 1 ml 10% sodium nitrite (a) to 100 ml with distilled water. Prepare fresh daily and preserve in a brown glass bottle.
   (a) Sodium nitrite, 10% aqueous; dissolve 10 gm granular sodium nitrite ($NaNO_2$—Mallinckrodt, A.C.S.) in distilled water and dilute to 100 ml. Preserve in refrigerator.
5. **Ammonium Sulfamate, 0.5% Aqueous.** Dissolve 0.5 gm crystalline ammonium sulfamate ($NH_2SO_3NH_4$—Reagent Chemicals, Special) in distilled water and dilute to 100 ml. Stable indefinitely; discard if mold-growth occurs.
6. **N-(1-Naphthyl)-Ethylenediamine Dihydrochloride, 0.1% Aqueous (Bratton-Marshall).** Dissolve 100 mg N-(1-naphthyl)-ethylenediamine dihydrochloride ($C_{10}H_7HNCH_2CH_2NH_2 \cdot 2HCl$—Eastman #4835) in 100 ml distilled water. Preserve in a brown glass bottle in refrigerator. Stability about one month. Discard when a brownish color appears.

Technic

Note: For concentrations of free p-aminohippuric acid in excess of 7 mg per 100 ml, dilute the protein-free filtrate with an equal volume of distilled water before proceeding with Step 6 and multiply the values obtained from the calibration table by 2.

1. In a 30 ml Erlenmeyer flask place
   10 ml distilled water
   1 ml sample from an Ostwald-Folin pipette, thoroughly rinsing out the pipette by drawing in and expelling some of the mixture several times.
2. Add 3 ml acid cadmium sulfate solution (1); mix thoroughly by a rotatory motion and allow to stand for 1 minute.
3. Add 1 ml 1.1 N sodium hydroxide (2) while constantly agitating the flask.

4. Stopper the flask with a clean rubber stopper, shake vigorously until foam-free and allow to stand for 10 minutes.
5. Centrifuge at 3000 rpm for 5 minutes and decant the supernatant fluid through a small filter paper into a dry test tube.
6. In a 10 ml graduated test tube (sample) place
   5 ml protein-free filtrate.
7. In another 10 ml graduated test tube (reagent blank) place
   5 ml distilled water.
8. To each tube add
   1 ml 1.2 N hydrochloric acid (3) and mix
   0.5 ml 0.1% sodium nitrite (4) and mix.
9. Allow to stand for not less than 3 minutes nor more than 5 minutes and add 0.5 ml 0.5% ammonium sulfamate (5) and mix.
10. Allow to stand for not less than 3 minutes nor more than 5 minutes and add 0.5 ml 0.1% N-(1-naphthyl)-ethylenediamine dihydrochloride (6) and mix.
11. Allow to stand at room temperature away from direct light for 10 minutes and adjust volume to exactly 10 ml with distilled water.
12. Measure the transmittancy of the sample against the reagent blank set at 100% transmission at a wave length of **540** mμ and obtain the concentration of free p-aminohippuric acid from the calibration table.

CALIBRATION PROCEDURE

Special Apparatus

Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.
Graduated test tubes—10 ml in 0.1 ml; the 10 ml graduation must be accurately calibrated.

Reagents

3. **Hydrochloric Acid, Volumetric 1.2 N.** Same as for Test Procedure.
4. **Sodium Nitrite, 0.1% Aqueous.** Same as for Test Procedure.
5. **Ammonium Sulfamate, 0.5% Aqueous.** Same as for Test Procedure.
6. **N-(1-Naphthyl)-Ethylenediamine Dihydrochloride, 0.1% Aqueous (Bratton-Marshall).** Same as for Test Procedure.
7. **p-Aminohippuric Acid Standards**
   (a) p-Aminohippuric acid stock standard (1 ml contains 1 mg p-aminohippuric acid). Dry p-aminohippuric acid ($NH_2C_6H_4CO$-$NHCH_2COOH$—Eastman #5704) at 90°C. for 18 hours; transfer 100 mg to a 100 ml volumetric flask; add approximately 80 ml distilled water and shake until dissolved; dilute to volume. Preserve in refrigerator.
   (b) Dilute standard (1 ml contains 0.04 mg p-aminohippuric acid). In a 100 ml volumetric flask place 4 ml stock standard (7a) and dilute to volume with distilled water. Prepare just before use.
   (c) Calibration standards. In a series of six accurately calibrated 50 ml volumetric flasks place respectively 0, 1, 2, 4, 5 and 6 ml of the dilute standard (7b) and dilute to volume with distilled water. These standards represent concentrations of p-aminohippuric acid equivalent to 0, 1.2, 2.4, 4.8, 6.0 and 7.2 mg per 100 ml sample. Prepare just before use.

Technic

1. Transfer 5 ml of each calibration standard (7c) to a correspondingly marked 10 ml graduated test tube.
2. Proceed exactly as outlined in the Test Procedure, Steps 8–11, inclusive.
3. Set the zero concentration standard at 100% transmission at a wave length of **540** mμ and record the transmittancy of the 1.2, 2.4, 4.8, 6.0 and 7.2 mg per cent standards. The transmittancy of the 7.2 mg standard should be approximately 19%.
4. Plot the observed values on semi-log graph paper—the points fall on a straight line which passes through the origin (Calibration Curve A-25).
5. Prepare a calibration table from the T-C values obtained from the plotted curve.

Method A-25b

DETERMINATION IN URINE

SAMPLE

Aliquot of a 24-hour specimen. For clearance test—individual specimens according to the technic followed. Keep cool until use.

TEST PROCEDURE

Special Apparatus

As listed under the Test Procedure for blood.

Reagents

1. **Acid Cadmium Sulfate Solution (Smith et al.)** Same as for blood.
2. **Sodium Hydroxide, Volumetric 1.1 N.** Same as for blood.
3. **Hydrochloric Acid, Volumetric 1.2 N.** Same as for blood.
4. **Sodium Nitrite, 0.1% Aqueous.** Same as for blood.

5. **Ammonium Sulfamate, 0.5% Aqueous.** Same as for blood.
6. **N-(1-Naphthyl)-Ethylenediamine Dihydrochloride, 0.1% Aqueous (Bratton-Marshall).** Same as for blood.

Technic

Note: For samples which contain no protein, increase the dilution in Step 2 tenfold, eliminate Steps 3–7 inclusive and proceed as outlined.

1. Filter a small portion of thoroughly mixed urine.
2. Dilute such an amount of filtered urine to a definite volume with distilled water so that the concentration of p-aminohippuric acid is between 1 and 6 mg per 100 ml.
3. In a 30 ml Erlenmeyer flask place
   10 ml distilled water
   1 ml diluted urine from an Ostwald-Folin pipette, thoroughly rinsing out the pipette by drawing in and expelling some of the mixture several times.
4. Add 3 ml acid cadmium sulfate solution (1); mix thoroughly by a rotatory motion and allow to stand for 1 minute.
5. Add 1 ml 1.1 N sodium hydroxide (2) while constantly agitating the flask.
6. Stopper the flask with a clean rubber stopper, shake vigorously and allow to stand for 10 minutes.
7. Centrifuge at 3000 rpm for 5 minutes and decant the supernatant fluid through a small filter paper into a dry test tube.
8. Proceed exactly as outlined in the Test Procedure for blood, Steps 6–11 inclusive.
9. Measure the transmittancy of the sample against the reagent blank set at 100% transmission at a wave length of **540** mμ and multiply the value from the calibration table for blood by the proper correction factor for the dilution made in Step 2 to obtain the concentration of free p-aminohippuric acid in mg per 100 ml urine.

PROCEDURE FOR USE WITH LINEAR-SCALE PHOTOELECTRIC COLORIMETERS (SHEARD-SANFORD TYPE)

TEST PROCEDURE

Exactly as outlined; set the reagent blank at 100 and use a green filter.

CALIBRATION PROCEDURE

Exactly as outlined—use a green filter; plot the observed readings on semi-log graph paper—the points fall on a straight line passing through the origin.

PROCEDURE FOR USE WITH LOGARITHMIC-SCALE PHOTOELECTRIC COLORIMETERS (KLETT-SUMMERSON TYPE)

TEST PROCEDURE

Exactly as outlined; set the reagent blank at 0 and use a green filter.

CALIBRATION PROCEDURE

Exactly as outlined—use a green filter; plot the observed readings on linear graph paper—the points fall on a straight line passing through the origin.

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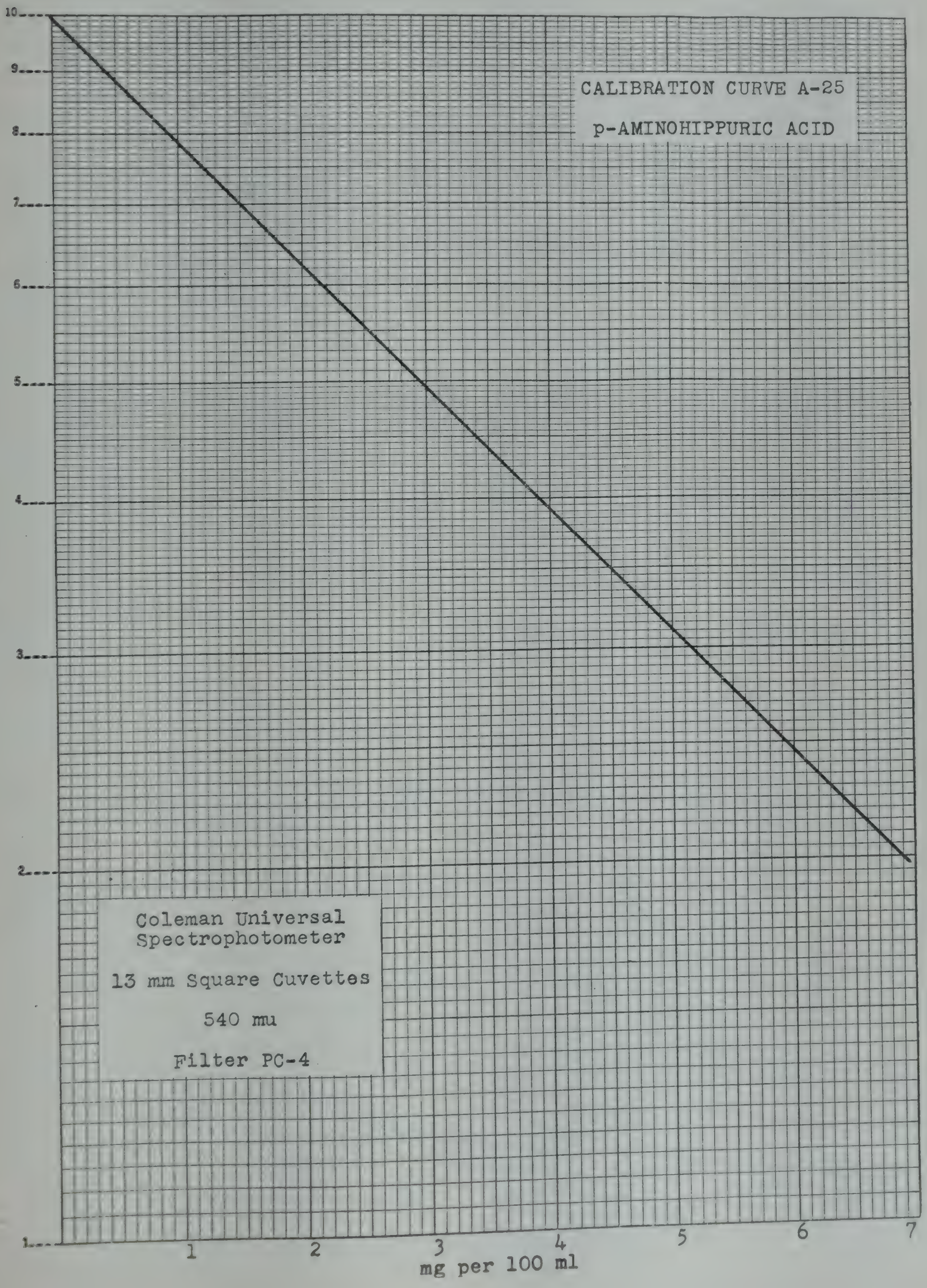
NOTES


CALIBRATION CURVE A-25
p-AMINOHIPPURIC ACID
Coleman Universal Spectrophotometer
13 mm Square Cuvettes
540 mu
Filter PC-4
10
9
8
7
6
5
4
3
2
1
1
2
3
4
5
6
7
mg per 100 ml

Meter Reading	0	1	2	3	4	5	6	7	8	9
0										
10										
20										
30										
40										
50										
60										
70										
80										
90										

p-AMINOSALICYLIC ACID

A spectrophotometric adaptation based on the method of Tennent and Leland (1949)

PRINCIPLE

p-Aminosalicylic acid is treated with excess nitrous acid and coupled with diazotized p-nitroaniline in the presence of pyridine to form a blue color. When the order of addition of the reagents is reversed, nitrous acid is destroyed and a red color is produced. By developing the red color in one aliquot of sample and the blue color in another, the difference between the color densities is directly proportional to the concentration of p-aminosalicylic acid. Since colors produced by interfering substances have the same intensity in both reactions, their effect is eliminated by the use of two aliquots, thus rendering a blank determination unnecessary.

DETERMINATION IN WHOLE BLOOD

SAMPLE

Blood withdrawn during post-absorptive state. Anticoagulant—potassium oxalate. The presence of abnormally high concentrations of uric acid inhibits the color reactions.

TEST PROCEDURE

Special Apparatus

Graduated centrifuge tubes, 15 ml; the 10 ml graduation must be accurately calibrated.
Ostwald-Folin pipettes accurately calibrated "to contain" 1 ml.
Graduated test tubes—10 ml in 0.1 ml.
Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.

Reagents

1. **Ethyl Alcohol, 95%** (C_2H_5OH—U. S. Industrial Chemicals, U.S.P.).
2. **Pyridine** ($CH(C_2H_2)_2N$—Baker & Adamson, Reagent). The pyridine must be refluxed for several hours with potassium permanganate and redistilled; collect the distillate in small fractions and use only those fractions which give a negative or barely perceptible blank.
3. **Coupling Reagent (Tennent-Leland).** Just prior to use mix 1 ml 2% sodium nitrite (a), 8 ml 0.02% p-nitroaniline (b) and 1 ml concentrated hydrochloric acid (HCl—Baker & Adamson, A.C.S.).
   (a) Sodium nitrite, 2% aqueous; dissolve 2 gm granular sodium nitrite ($NaNO_2$—Mallinckrodt, A.C.S.) in distilled water and dilute to 100 ml. Preserve in refrigerator. Prepare fresh weekly.
   (b) p-Nitroaniline, 0.02% aqueous; dissolve 20 mg p-nitroaniline ($NO_2C_6H_4NH_2$—Eastman #179) in 100 ml distilled water with the aid of gentle heat. Preserve in refrigerator. Prepare fresh weekly.
4. **Sodium Hydroxide, Volumetric 5 N.** Prepare by diluting slightly more than the required amount of concentrated carbonate-free sodium hydroxide solution (see Appendix); dilute 10 ml of this approximately 5 N solution to 100 ml; titrate against standard 0.5 N acid using phenolphthalein as indicator; adjust accordingly and re-titrate—20 ml of 0.5 N acid require 20 ml of a 1:10 dilution of 5 N alkali. Preserve in ceresin-lined bottle.

Technic

Note: For concentrations of p-aminosalicylic acid in excess of 20 mg per 100 ml, dilute the final colored solutions in Step 12 with an equal volume of distilled water; repeat Step 13 and multiply the values obtained from the calibration table by 2.

1. In a 15 ml graduated centrifuge tube place
   4 ml distilled water
   1 ml blood from an Ostwald-Folin pipette, thoroughly rinsing out the pipette by drawing in and expelling some of the mixture several times.
2. Mix and allow to stand until the blood is completely laked.
3. Dilute to exactly 10 ml with 95% ethyl alcohol (1).
4. Centrifuge at 1500 rpm for 3 minutes.
5. In each of two 10 ml graduated test tubes (marked "A" and "B") place 1 ml clear centrifugate.
6. Place tubes in a water bath at 20° C. for 5 minutes.
7. To each tube add 3 ml distilled water and mix.
8. To tube "A" add in the order named
   1 ml pyridine (2) and mix
   1 ml coupling reagent (3) and mix.
9. To tube "B" add in the order named
   1 ml coupling reagent (3) and mix
   1 ml pyridine (2) and mix.
10. Replace tubes in water bath at 20° C. for 5 minutes.
11. To each tube add 0.5 ml 5 N sodium hydroxide (4) and mix thoroughly.
12. Replace tubes in water bath at 20° C. for 20 minutes.
13. Measure the transmittancy of tube "B" (blue color) against tube "A" (red color) set at 100% transmission at a wave length of 620 mμ and obtain the concentration of p-aminosalicylic acid from the calibration table.

CALIBRATION PROCEDURE

Note: The calibration curve must be checked with each new lot of pyridine. It is advisable to run a standard simultaneously with the unknown.

Special Apparatus

Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.
Graduated test tubes—10 ml in 0.1 ml.

Reagents

2. **Pyridine.** Same as for Test Procedure.
3. **Coupling Reagent (Tennent-Leland).** Same as for Test Procedure.
4. **Sodium Hydroxide, Volumetric 5 N.** Same as for Test Procedure.
5. **p-Aminosalicylic Acid Standards**
    - (a) p-Aminosalicylic acid stock standard (1 ml contains 1 mg p-aminosalicylic acid). In a 100 ml volumetric flask place 100 mg p-aminosalicylic acid ($2\text{-HONH}_2\text{C}_6\text{H}_3\text{COOH}$—Reagent Chemicals, Special); add approximately 80 ml distilled water and 0.5 ml 5 N sodium hydroxide (4); shake until dissolved, with the aid of gentle heat if necessary; dilute to volume. Preserve in refrigerator.
    - (b) Dilute standard (1 ml contains 0.02 mg p-aminosalicylic acid). In a 100 ml volumetric flask place 2 ml stock standard (5a) and dilute to volume with distilled water. Prepare just before use.
    - (c) Calibration standards. In a series of six accurately calibrated 10 ml volumetric flasks place respectively 0, 2, 4, 6, 8 and 10 ml of the dilute standard (5b) and dilute to volume with distilled water. These standards represent concentrations of p-aminosalicylic acid equivalent to 0, 4, 8, 12, 16 and 20 mg per 100 ml sample. Prepare just before use.

Technic

1. Set up a series of six correspondingly marked 10 ml graduated test tubes—label the tubes in this series "A."
2. Set up another series of six correspondingly marked 10 ml graduated test tubes—label the tubes in this series "B."
3. Transfer 1 ml of each calibration standard (5c) to the appropriate "A" and "B" tubes.
4. Place tubes in a water bath at 20° C. for 5 minutes.
5. To each tube add 3 ml distilled water and mix.
6. To each "A" tube add in the order named
    1 ml pyridine (2) and mix
    1 ml coupling reagent (3) and mix.
7. To each "B" tube add in the order named
    1 ml coupling reagent (3) and mix
    1 ml pyridine (2) and mix.
8. Proceed exactly as outlined in the Test Procedure, Steps 10–12 inclusive.
9. Measure the transmittancy of each "B" tube against the corresponding "A" tube set at 100% transmission at a wave length of **620** mμ and record. The transmittancy of the 20 mg standard should be approximately 29%.

Note: With the zero concentration "A" tube set at 100% transmission at a wave length of **620** mμ, the transmittancy of the zero concentration "B" tube should be very close to 100% (never less than 99%).

10. Plot the observed values on semi-log graph paper—the points fall on a straight line which passes through the origin (Calibration Curve A-28).
11. Prepare a calibration table from the T-C values obtained from the plotted curve.

Method A-28b

DETERMINATION IN URINE

SAMPLE

Aliquot of a 24-hour specimen or individual specimens as indicated. Keep cool during collection.

TEST PROCEDURE

Special Apparatus

Graduated test tubes—10 ml in 0.1 ml.
Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.

Reagents

2. **Pyridine.** Same as for blood.
3. **Coupling Reagent (Tennent-Leland).** Same as for blood.
4. **Sodium Hydroxide, Volumetric 5 N.** Same as for blood.

Technic

Note: For concentrations of p-aminosalicylic acid in excess of 200 mg per 100 ml, use a 200 ml volumetric flask in Step 2 and multiply the values obtained from the calibration table for blood by 20.

1. Filter a small portion of thoroughly mixed urine.
2. In a 100 ml volumetric flask place 1 ml filtered urine and dilute to volume with distilled water.
3. In each of two 10 ml graduated test tubes (marked "A" and "B") place 1 ml diluted urine.
4. Proceed exactly as outlined in the Test Procedure for blood, Steps 6–12 inclusive.
5. Measure the transmittancy of tube "B" (blue color) against tube "A" (red color) set at 100% transmission at a wave length of **620** mμ and multiply the value from the calibration table for blood by 10 to obtain the concentration of p-aminosalicylic acid in mg per 100 ml urine.

PROCEDURE FOR USE WITH LINEAR-SCALE PHOTOELECTRIC COLORIMETERS (SHEARD-SANFORD TYPE)

TEST PROCEDURE

Exactly as outlined; set the "A" tube at 100 and use a filter with a maximum transmission at **620** mμ.

CALIBRATION PROCEDURE

Exactly as outlined—use a filter with a maximum transmission at **620** mμ; plot the observed readings on semi-log graph paper—the points fall on a straight line passing through the origin.

PROCEDURE FOR USE WITH LOGARITHMIC-SCALE PHOTOELECTRIC COLORIMETERS (KLETT-SUMMERSON TYPE)

TEST PROCEDURE

Exactly as outlined; set the "A" tube at 0 and use a filter with a maximum transmission at **620** mμ.

CALIBRATION PROCEDURE

Exactly as outlined—use a filter with a maximum transmission at **620** mμ; plot the observed readings on linear graph paper—the points fall on a straight line passing through the origin.

Note: For instruments with cells, cuvettes or tubes requiring a final volume of more than 6.5 ml, follow the procedure as outlined and add 2 ml distilled water just before measurements are made in both the test and calibration procedures.

BIBLIOGRAPHY

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NOTES

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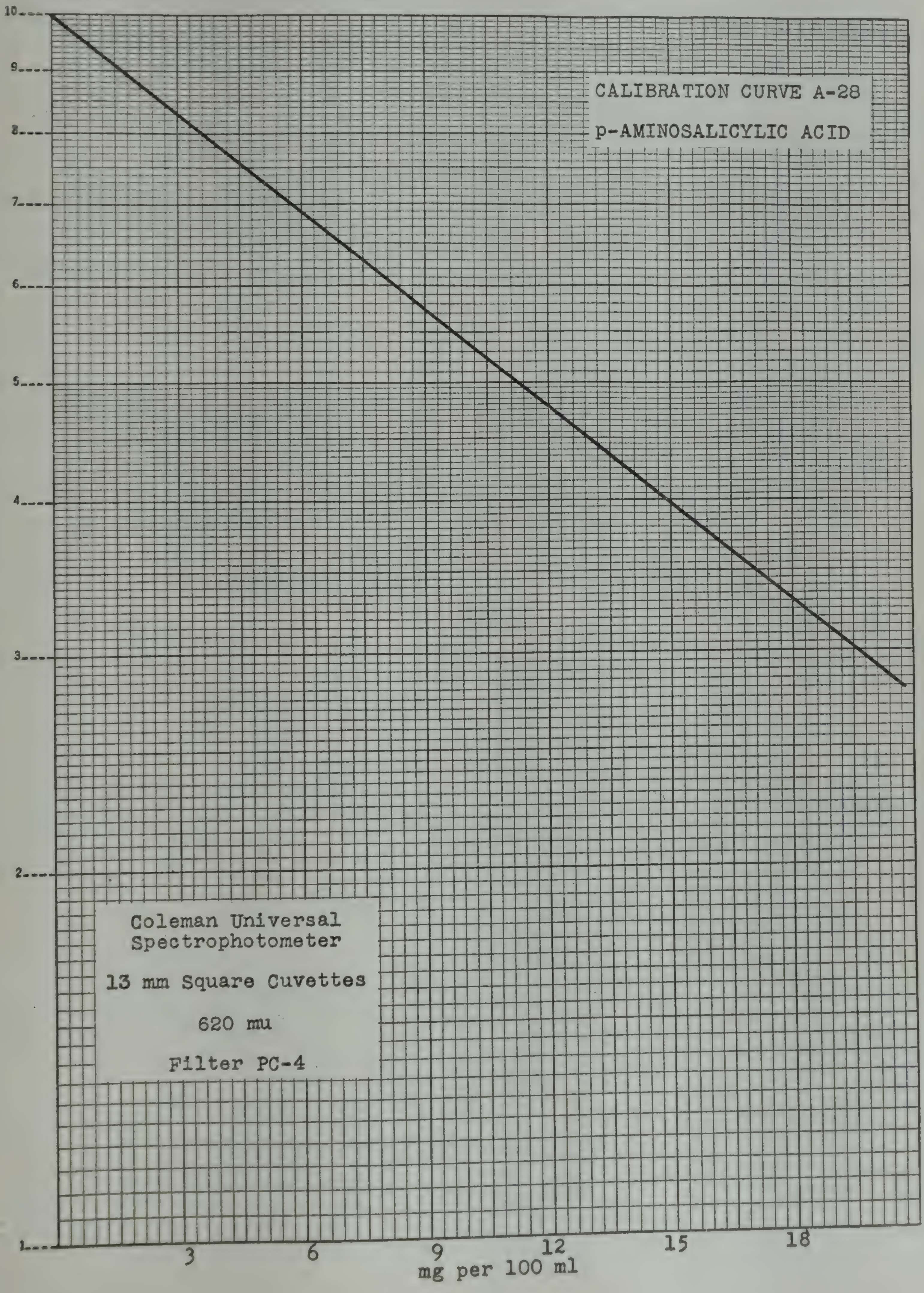
CALIBRATION CURVE A-28
p-AMINOSALICYLIC ACID
Coleman Universal Spectrophotometer
13 mm Square Cuvettes
620 mu
Filter PC-4
10
9
8
7
6
5
4
3
2
1
3
6
9
12
15
18
mg per 100 ml

Meter Reading	0	1	2	3	4	5	6	7	8	9
0										
10										
20										
30										
40										
50										
60										
70										
80										
90										

AMMONIA NITROGEN

A spectrophotometric adaptation based on the methods and modifications of Nash and Benedict (1921), Orr (1924), Murray (1925), Folin (1932), Van Slyke and Hiller (1933), Conway and Byrne (1933), Borsook (1935), Borsook and Dubnoff (1939), Conway (1940) and Russell (1944)

PRINCIPLE

Ammonia is liberated from the sample by sodium borate and is transferred by diffusion in a Conway unit into a sulfuric acid receiving solution. Upon treatment with phenol and hypochlorite in alkaline solution (Berthelot reaction), an intense blue color is developed.

DETERMINATION IN WHOLE BLOOD

SAMPLE

Blood withdrawn during post-absorptive state. Anticoagulant—potassium oxalate; only the special recrystallized salt (Reagent #1) should be used. Needles, syringes and collection tubes must not be contaminated by ammonia or its salts; the most rigid precautions must be taken to prevent contamination throughout the entire procedure. Sample tubes should be kept tightly covered at all times. The analysis should be carried out as soon as possible after withdrawal of the blood; samples should be stored in the refrigerator until use.

TEST PROCEDURE

Special Apparatus

Conway units consisting of a Pyrex glass dish with an inner circular chamber 40 mm in diameter and 5 mm high with a 70 mm square glass cover.

Note: Units should be thoroughly cleaned and rinsed under hot water—use no soap or acid cleaning solutions; rinse in ammonia-free distilled water (Reagent #2) and allow to dry in an inverted position—do not use cloth to dry the inside of the units. Units may be stored in a solution of 0.001 N hydrochloric acid and rinsed with ammonia-free distilled water just before use, removing any droplets from the inner wall with filter paper.

Ostwald-Folin pipettes accurately calibrated "to deliver" 2 ml.

Rapid delivery pipettes, 1 ml.

Graduated test tubes—10 ml in 0.1 ml; the 6 ml graduation must be accurately calibrated.

Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.

Reagents

1. **Potassium Oxalate, Recrystallized.** In a 600 ml Pyrex beaker place 200 ml ammonia-free distilled water (Reagent #2) and heat to boiling; while stirring constantly, add finely pulverized crystalline potassium oxalate ($K_2C_2O_4 \cdot H_2O$—J. T. Baker, A.C.S.) until the solution is completely saturated; immediately filter the hot solution through an ammonia-free paper into a 400 ml beaker; cover the beaker and allow to stand until cold; stir up the crystalline deposit, transfer to a sintered glass funnel (coarse porosity) and suck dry; wash twice with ice-cold ammonia-free distilled water; dry over sulfuric acid in vacuo.
2. **Water, Distilled, Ammonia-Free.** In an all-glass Pyrex distillation apparatus place 1 gm sodium carbonate (Na_2CO_3—Baker & Adamson, A.C.S.) and 1 gm potassium permanganate ($KMnO_4$—Baker & Adamson, A.C.S.); add 500 ml distilled water; redistil, rejecting the first 100 ml portion and collecting only the next 300 ml. Use for the preparation of all reagents and standards and where indicated in the test procedure.
3. **Sulfuric Acid, Volumetric 0.02 N.** Dilute 20 ml 0.1 N sulfuric acid (a) to 100 ml with ammonia-free distilled water (2). Prepare frequently.
   (a) Sulfuric acid, volumetric 0.1 N; add 3 ml concentrated sulfuric acid (H_2SO_4—Grasselli, A.C.S.) to 1000 ml ammonia-free distilled water (2) and mix; standardize against 0.1 N alkali using phenolphthalein as indicator.
4. **Alkaline Glycerine Sealing Mixture (Borsook-Dubnoff).** Add just sufficient concentrated sodium hydroxide solution to glycerine (reagent grade not necessary) to produce a pink color with phenolphthalein. Note: This sealing mixture is preferable to vaseline-paraffin mixtures because it is readily removed by simple washing.
5. **Borate Buffer Solution (Borsook-Dubnoff).** Just prior to use, mix 2 volumes concentrated borate buffer solution (a) with 1 volume 95% ethyl alcohol (C_2H_5OH—U. S. Industrial Chemicals, U.S.P.).
   (a) Borate buffer solution, concentrated; in a 1000 ml volumetric flask place 12.4 gm powdered boric acid (H_3BO_3—Baker & Adamson, A.C.S.); add approximately 800 ml ammonia-free distilled water (2) and shake until dissolved; add 20 ml 5 N sodium hydroxide (Reagent #7a) and mix; check the pH potentiometrically and adjust to pH 10.3 by adding more hydroxide; dilute to volume with ammonia-free distilled water and mix thoroughly. Preserve in ceresin-lined bottle.
6. **Manganous Chloride, 0.003 M.** Dissolve 59.4 mg crystalline manganous chloride ($MnCl_2 \cdot 4H_2O$—Baker & Adamson, Reagent) in 100 ml ammonia-free distilled water (2).
7. **Alkaline Phenol Reagent (Russell).** In a 100

ml mixing cylinder place 25 gm crystalline phenol (C_6H_5OH—Baker & Adamson, Acid Carbolic, Reagent, Loose Crystals); add 10 ml ammonia-free distilled water (2) and 54 ml 5 N sodium hydroxide (a); stopper and shake until solution is complete; dilute to volume with ammonia-free distilled water. Preserve in an amber resistant-glass bottle in refrigerator.

(a) Sodium hydroxide, volumetric 5 N; prepare by diluting slightly more than the required amount of concentrated carbonate-free sodium hydroxide solution (see Appendix); dilute 10 ml of this approximately 5 N solution to 100 ml; titrate against standard 0.5 N acid using phenolphthalein as indicator; adjust accordingly and re-titrate—20 ml of 0.5 N acid require 20 ml of a 1:10 dilution of 5 N alkali. Preserve in ceresin-lined bottle.

8. **Hypochlorite Solution (Russell).** In a 600 ml Pyrex beaker place 300 ml ammonia-free distilled water (2) and heat to 75° C.; add 25 gm finely pulverized calcium hypochlorite (CaOCl—J. T. Baker, U.S.P.) and dissolve as much as possible by vigorous stirring; add 135 ml cool, freshly boiled 20% potassium carbonate (a) while stirring; mix thoroughly; heat briefly to 90° C.; cool to room temperature; transfer to a 500 ml mixing cylinder and dilute to volume with ammonia-free distilled water. This solution must now be tested for the presence of calcium: filter a few ml of the solution; add 0.5 ml 20% potassium carbonate (a) to 1 ml of the filtered solution and heat in boiling water for 3 minutes—the solution must remain crystal clear; if calcium is present, as denoted by a cloudy solution, add more 20% potassium carbonate to the original solution and repeat the test for calcium; this process must be repeated until the test for calcium is negative. Filter the calcium-free solution through a Whatman #40 paper and determine the amount of free chlorine as follows: place 10 ml distilled water in a small titration flask; add 2 ml of the clear, filtered hypochlorite solution, 2 ml freshly prepared 5% potassium iodide and 1 ml glacial acetic acid; titrate against volumetric 0.1 N sodium thiosulfate using starch as indicator—2 ml of the hypochlorite solution must require 7.5–8.0 ml of the thiosulfate indicating a free chlorine content of 1.3–1.4 gm per 100 ml; if necessary, adjust accordingly and re-titrate. The determination of free chlorine should be made at weekly intervals. Preserve in small, completely filled brown glass bottles (not more than 4 oz capacity) in refrigerator.

(a) Potassium carbonate, 20% aqueous; dissolve 20 gm anhydrous potassium carbonate (K_2CO_3—Baker & Adamson, A.C.S.) in 200 ml ammonia-free distilled water (2); boil down rapidly to one-half volume; add 50 ml ammonia-free distilled water and again boil down to half volume; cover the beaker and allow to stand in an ammonia-free atmosphere until cool; dilute to 100 ml with ammonia-free distilled water. Preserve in resistant-glass bottle.

Technic

Note: It is essential that all operations be carried out in an ammonia-free atmosphere.

1. To one side of the outer compartment of a Conway unit (sample) place
   2 ml oxalated whole blood from an Ostwald-Folin pipette.
2. In the outer compartment of another Conway unit (reagent blank) place
   2 ml ammonia-free distilled water (2).
3. In the inner compartment of each unit place 1 ml 0.02 N sulfuric acid (3).
4. Dip a glass stirring rod into the alkaline glycerine sealing mixture (4) and apply a coating of the mixture to the rims of the units, i.e., the upper edges of the outer walls; thinly coat the under surfaces of the covers with the sealing mixture.
5. Place the covers on the units, coated side down, and then slide the covers aside just far enough to allow entry of a pipette tip at the highest point of the unit and on the opposite side from where the fluid in the outer chamber has been placed.
6. Insert the tip of a rapid delivery pipette through the opening and quickly and carefully, avoiding spattering, add 1 ml borate buffer solution (5) to the outer compartment of each unit and slide covers in place immediately—make certain that the covers are firmly sealed to the units by stretching a broad rubber band over the cover and under the bottom of the unit.
7. Thoroughly mix the fluids in the outer compartments by slightly tilting and rotating the units for about 15 seconds—do not allow any of the fluid in one cell to enter the other.
8. Place the units in the incubator at 38° C. (never more than 40° C.) for 90 minutes (or allow to stand at room temperature for 120 minutes)—the formation of dew on the under surface of the covers is of no consequence.
9. Slide off the covers and, by means of small pipettes fitted with rubber bulbs, transfer the contents of the center well of each unit to a correspondingly marked 10 ml graduated test tube, washing out the wells with five 0.2 ml portions ammonia-free distilled water (2) and transferring the washings to the appropriate test tube.
10. Place the tubes in ice-cold water.
11. To each tube add
    1 drop 0.003 M manganous chloride (6) and mix
    1 ml cold alkaline phenol reagent (7) and mix
    0.5 ml cold hypochlorite solution (8) and mix thoroughly by gentle rotation in the ice bath.

12. Immediately place the tubes in boiling water for 5 minutes—the color in the sample tube is green.
13. Cool under running tap water to room temperature—the green color changes to blue.
14. Dilute to 6 ml with ammonia-free distilled water (2) and mix.
15. Measure the transmittancy of the sample against the reagent blank set at 100% transmission at a wave length of **625 mμ** and obtain the concentration of ammonia nitrogen from the calibration table for blood.

CALIBRATION PROCEDURE FOR BLOOD

Special Apparatus

Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.
Conway units, as described under the Test Procedure.
Rapid delivery pipettes, 1 ml.
Graduated test tubes—10 ml in 0.1 ml; the 6 ml graduation must be accurately calibrated.

Reagents

2. **Water, Distilled, Ammonia-Free.** Same as for Test Procedure.
3. **Sulfuric Acid, Volumetric 0.02 N.** Same as for Test Procedure.
4. **Alkaline Glycerine Sealing Mixture (Borsook-Dubnoff).** Same as for Test Procedure.
5. **Borate Buffer Solution (Borsook-Dubnoff).** Same as for Test Procedure.
6. **Manganous Chloride, 0.003 M.** Same as for Test Procedure.
7. **Alkaline Phenol Reagent (Russell).** Same as for Test Procedure.
8. **Hypochlorite Solution (Russell).** Same as for Test Procedure.
9. **Ammonia Standards**
   (a) Nitrogen stock standard (1 ml contains 1 mg nitrogen). Dry pyridine-free ammonium sulfate ($(NH_4)_2SO_4$—Reagent Chemicals, Special) at 110° C. for 1 hour; transfer 471.6 mg to a 100 ml volumetric flask; add approximately 50 ml ammonia-free distilled water (2); shake until dissolved and dilute to volume with same. Preserve with a few drops of toluene. Stability indefinite.
   (b) Dilute standard (1 ml contains 0.01 mg nitrogen). In a 100 ml volumetric flask place 1 ml stock standard (9a) and dilute to volume with ammonia-free distilled water (2). Prepare just before use.
   (c) Calibration standards. In a series of four accurately calibrated 10 ml volumetric flasks place respectively 0, 1, 2 and 3 ml of the dilute standard (9b) and dilute to volume with ammonia-free distilled water (2). These standards represent concentrations of ammonia nitrogen equivalent to 0, 0.1, 0.2 and 0.3 mg per 100 ml sample. Prepare just before use and proceed immediately as follows.

Technic

1. Transfer 2 ml of each calibration standard (9c) to the outer compartment of correspondingly marked Conway units.
2. Proceed exactly as outlined in the Test Procedure, Steps 3–14 inclusive.
3. Set the zero concentration standard at 100% transmission at a wave length of **625 mμ** and record the transmittancy of the 0.1, 0.2 and 0.3 mg per cent standards. The transmittancy of the 0.3 mg standard should be approximately 32%.
4. Plot the observed values on semi-log graph paper—the points fall on a straight line which passes through the origin (Calibration Curve A-30A).
5. Prepare a calibration table from the T-C values obtained from the plotted curve.

Method A-30b

DETERMINATION IN URINE

SAMPLE

Aliquot of a 24-hour specimen. Collect the sample in a thoroughly clean, tightly covered container, adding 1 drop concentrated sulfuric acid for each 200 ml urine as voided. Keep cool during collection. The slightest decomposition vitiates the results—this is prevented by the addition of a small amount of toluene to the sample container. Analyses should not be unduly delayed.

TEST PROCEDURE

Special Apparatus

Conway units, as described under the Test Procedure for blood.
Ostwald-Folin pipettes accurately calibrated "to deliver" 1 ml.
Rapid delivery pipettes, 1 ml.
Graduated test tubes—10 ml in 0.1 ml; the 10 ml graduation must be accurately calibrated.
Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.

Reagents

2. **Water, Distilled, Ammonia-Free.** Same as for blood.
3. **Sulfuric Acid, Volumetric 0.02 N.** Same as for blood.
4. **Alkaline Glycerine Sealing Mixture (Borsook-Dubnoff).** Same as for blood.
5. **Borate Buffer Solution (Borsook-Dubnoff).** Same as for blood.
6. **Manganous Chloride, 0.003 M.** Same as for blood.
7. **Alkaline Phenol Reagent (Russell).** Same as for blood.
8. **Hypochlorite Solution (Russell).** Same as for blood.

Technic

Note: For concentrations of ammonia nitrogen in excess of 200 mg per 100 ml, dilute 1 ml filtered urine to 200 ml in Step 2 and multiply the values obtained from the calibration table for urine by 2.

1. Filter a small portion of thoroughly mixed urine.
2. In a 100 ml volumetric flask place 1 ml filtered urine and dilute to volume with ammonia-free distilled water (2).
3. To one side of the outer compartment of a Conway unit (sample) place 1 ml diluted urine from an Ostwald-Folin pipette.
4. In the outer compartment of another Conway unit (reagent blank) place 1 ml ammonia-free distilled water (2).
5. Proceed exactly as outlined in the Test Procedure for blood, Steps 3–10 inclusive.
6. To each tube add
   1 drop 0.003 M manganous chloride (6) and mix
   1 ml cold alkaline phenol reagent (7) and mix
   1 ml cold hypochlorite solution (8) and mix thoroughly by gentle rotation in the ice bath.
7. Immediately place the tubes in boiling water for 5 minutes—the color in the sample tube is green.
8. Cool under running tap water to room temperature—the green color changes to blue.
9. Dilute to 10 ml with ammonia-free distilled water (2) and mix.
10. Measure the transmittancy of the sample against the reagent blank set at 100% transmission at a wave length of **625 mμ** and obtain the concentration of ammonia nitrogen from the calibration table for urine.

Calculation

$$\frac{\text{Volume of 24-hour specimen}}{100} \times \text{Ammonia nitrogen in mg per 100 ml} = \text{Mg ammonia nitrogen excreted in 24 hours}$$

CALIBRATION PROCEDURE FOR URINE

Special Apparatus

Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.

Conway units, as described under the Test Procedure for blood.

Rapid delivery pipettes, 1 ml.

Graduated test tubes—10 ml in 0.1 ml; the 10 ml graduation must be accurately calibrated.

Reagents

2. **Water, Distilled, Ammonia-Free.** Same as for Test Procedure.
3. **Sulfuric Acid, Volumetric 0.02 N.** Same as for Test Procedure.
4. **Alkaline Glycerine Sealing Mixture (Borsook-Dubnoff).** Same as for Test Procedure.
5. **Borate Buffer Solution (Borsook-Dubnoff).** Same as for Test Procedure.
6. **Manganous Chloride, 0.003 M.** Same as for Test Procedure.
7. **Alkaline Phenol Reagent (Russell).** Same as for Test Procedure.
8. **Hypochlorite Solution (Russell).** Same as for Test Procedure.
10. **Ammonia Standards**
    (a) Nitrogen stock standard (1 ml contains 1 mg nitrogen). This is the same as Reagent #9a under the Calibration Procedure for blood.
    (b) Dilute standard (1 ml contains 0.04 mg nitrogen). In a 100 ml volumetric flask place 4 ml stock standard (10a) and dilute to volume with ammonia-free distilled water (2). Prepare just before use.
    (c) Calibration standards. In a series of six accurately calibrated 10 ml volumetric flasks place respectively 0, 1, 2, 3, 4 and 5 ml of the dilute standard (10b) and dilute to volume with ammonia-free distilled water (2). These standards represent concentrations of ammonia nitrogen equivalent to 0, 40, 80, 120, 160 and 200 mg per 100 ml sample. Prepare just before use and proceed immediately as follows.

Technic

1. Transfer 1 ml of each calibration standard (10c) to the outer compartment of correspondingly marked Conway units.
2. Proceed exactly as outlined in the Test Procedure for urine, Steps 5–9 inclusive.
3. Set the zero concentration standard at 100% transmission at a wave length of **625 mμ** and record the transmittancy of the 40, 80, 120, 160 and 200 mg per cent standards. The transmittancy

of the 200 mg standard should be approximately 18%.

4. Plot the observed values on semi-log graph paper—the points fall on a straight line which passes through the origin (Calibration Curve A-30B).
5. Prepare a calibration table from the T-C values obtained from the plotted curve.

PROCEDURE FOR USE WITH LINEAR-SCALE PHOTOELECTRIC COLORIMETERS (SHEARD-SANFORD TYPE)

TEST PROCEDURE

Exactly as outlined; set the reagent blank at 100 and use a filter with maximum transmission at **625** mμ.

CALIBRATION PROCEDURE

Exactly as outlined—use a filter with maximum transmission at **625** mμ; plot the observed readings on semi-log graph paper—the points fall on a straight line passing through the origin.

PROCEDURE FOR USE WITH LOGARITHMIC-SCALE PHOTOELECTRIC COLORIMETERS (KLETT-SUMMERSON TYPE)

TEST PROCEDURE

Exactly as outlined; set the reagent blank at 0 and use a filter with maximum transmission at **625** mμ.

CALIBRATION PROCEDURE

Exactly as outlined—use a filter with maximum transmission at **625** mμ; plot the observed readings on linear graph paper—the points fall on a straight line passing through the origin.

Note: For instruments with cells, cuvettes or tubes requiring a final volume of more than 6 ml, dilute the final colored solution to 10 ml in both the test and calibration procedures for blood.

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NOTES

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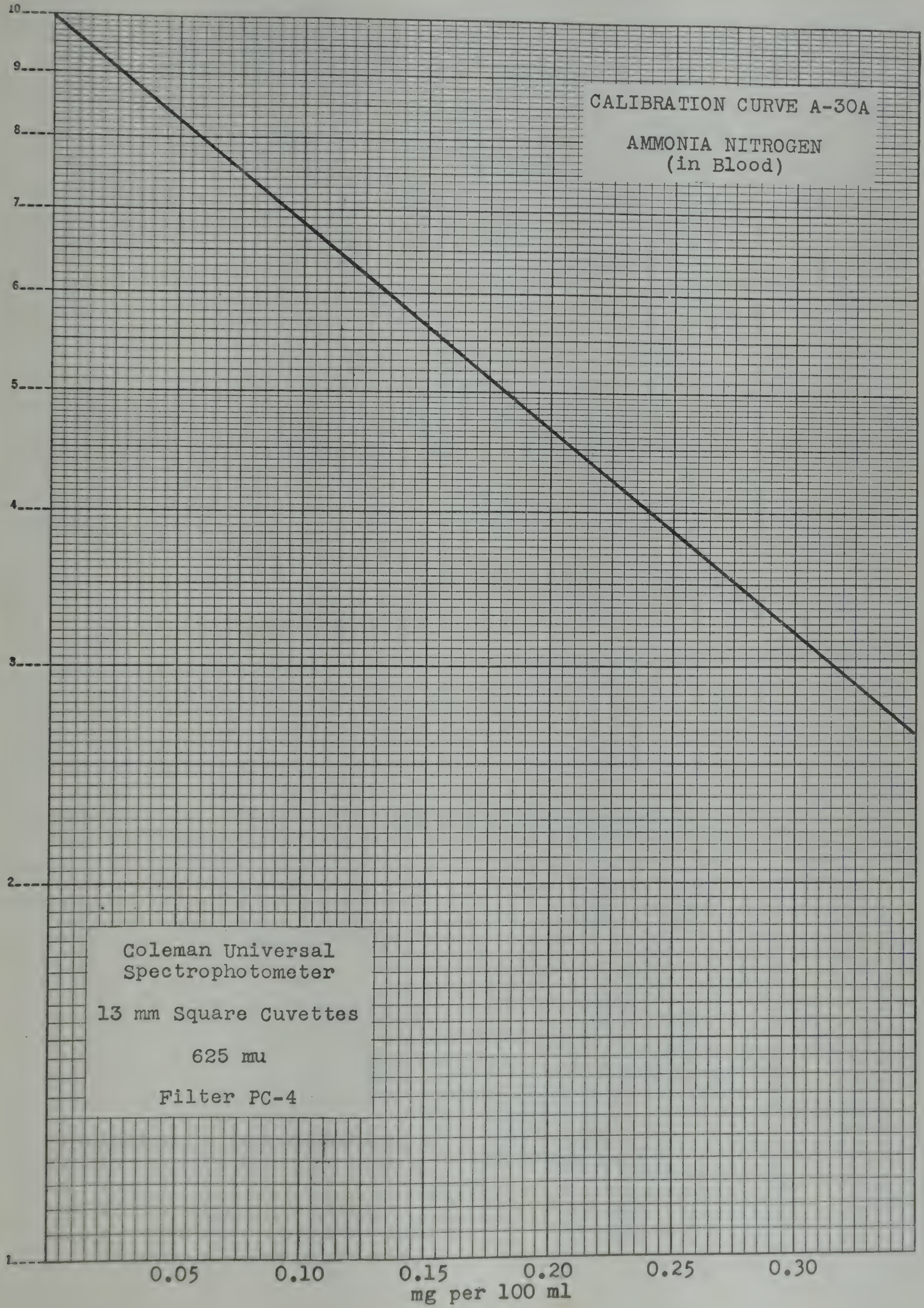
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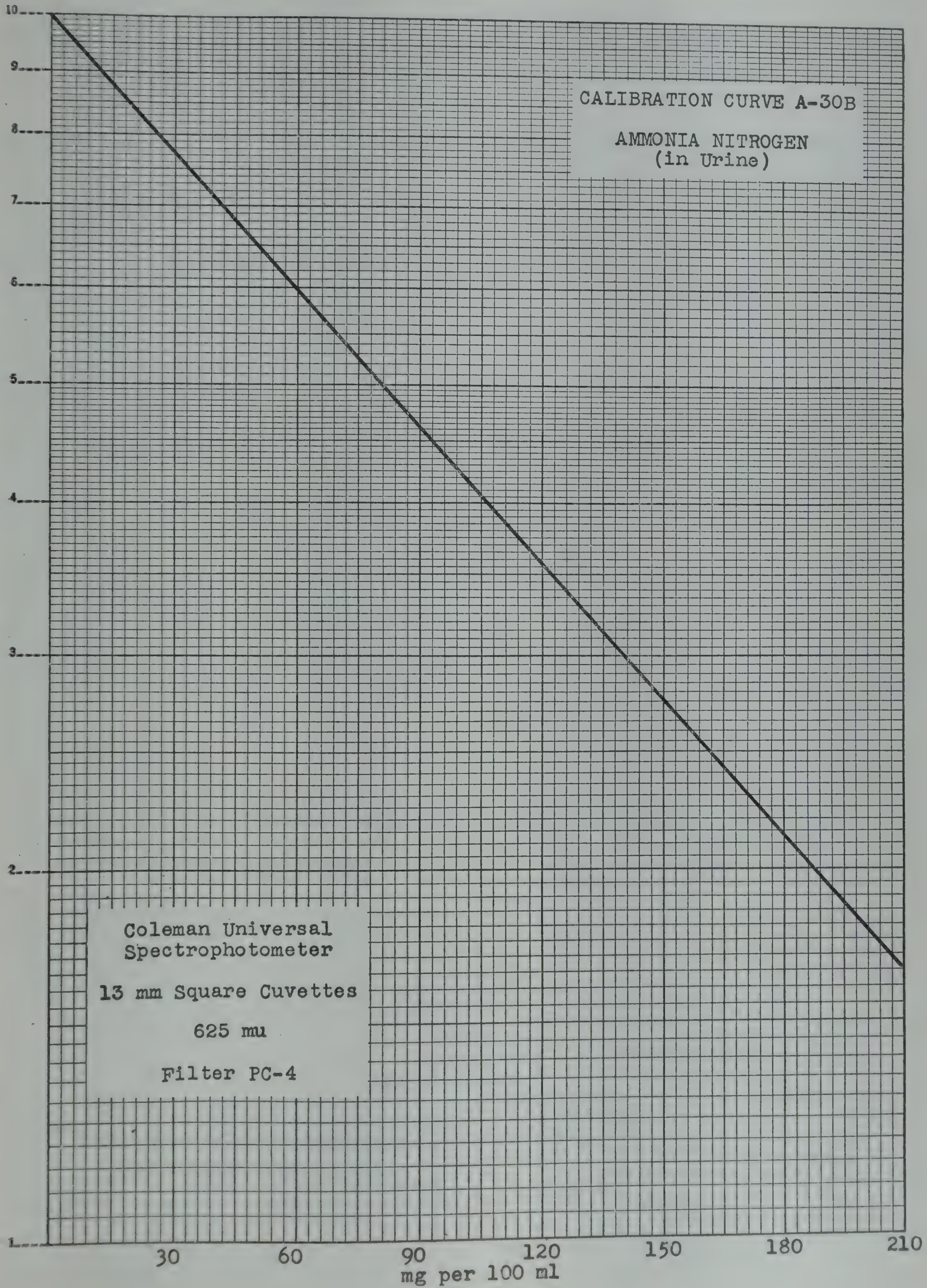
AMMONIA NITROGEN
(in Blood)
Coleman Universal
Spectrophotometer
13 mm Square Cuvettes
625 mu
Filter PC-4
10
9
8
7
6
5
4
3
2
1
0.05
0.10
0.15
0.20
0.25
0.30
mg per 100 ml

Meter Reading	0	1	2	3	4	5	6	7	8	9
0										
10										
20										
30										
40										
50										
60										
70										
80										
90										


CALIBRATION CURVE A-30B
AMMONIA NITROGEN
(in Urine)
Coleman Universal Spectrophotometer
13 mm Square Cuvettes
625 mu
Filter PC-4
10
9
8
7
6
5
4
3
2
1
30
60
90
120
150
180
210
mg per 100 ml

Meter Reading	0	1	2	3	4	5	6	7	8	9
0										
10										
20										
30										
40										
50										
60										
70										
80										
90										

AMYLASE

A spectrophotometric adaptation based on the methods and modifications of
Somogyi (1938; 1945), Nelson (1944) and Andersch (1946)

PRINCIPLE

Plasma or serum is incubated for a definite period with a b-amylose substrate, buffered at pH 6.8. The concentration of total reducing substances before and after digestion is determined by means of the alkaline copper-arsenomolybdate reaction as for true dextrose (Method D-12), the same analytical principles being involved. The difference between these two values (the reducing substances produced per 100 ml of enzyme in 15 minutes) is considered as the measure of the amylolytic power of the sample. The results are expressed in terms of amylase units, one unit being equivalent to 1 mg total reducing substances per 100 ml sample.

DETERMINATION IN PLASMA OR SERUM

SAMPLE

Plasma or serum from blood withdrawn at any time of the day but preferably at the height of an attack—analyses preferably made on serum. Anticoagulant for plasma—potassium oxalate. Carry out protein precipitation as soon as possible, although amylase activity, if high, is not significantly decreased in samples refrigerated overnight. The same type of sample, that is, either plasma or serum, must be used for the determination of total reducing substances before and after digestion.

TEST PROCEDURE

Special Apparatus

Ostwald-Folin pipettes accurately calibrated "to contain" 0.5 ml.

Ostwald-Folin pipettes accurately calibrated "to deliver" 1 ml.

Folin sugar tubes—the 4 ml level must fall within the constricted portion of the tube and the 25 ml graduation must be accurately calibrated. (For a more detailed description see Method D-10.)

Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.

Reagents

1. **Phosphate Buffer Solution, pH 6.8.** In a 1000 ml volumetric flask place 250 ml 0.2 M monobasic potassium phosphate (a) and 118 ml 0.2 M sodium hydroxide (b); mix and dilute to volume with freshly boiled distilled water. Adjust potentiometrically to pH 6.8 by the addition of either phosphate or hydroxide. Preserve in resistant-glass bottle.
   (a) Potassium phosphate, monobasic, 0.2 M; dry monobasic potassium phosphate (KH_2PO_4—Merck, Soerensen) at 110° C. to constant weight; dissolve 27.312 gm in freshly boiled distilled water and dilute to 1000 ml. Preserve in resistant-glass bottle.
   (b) Sodium hydroxide, 0.2 M; prepare by diluting slightly more than the required amount of concentrated carbonate-free sodium hydroxide solution (see Appendix); titrate against standard 0.1 N acid using phenolphthalein as indicator; adjust accordingly and re-titrate—20 ml of 0.1 N acid require 10 ml of 0.2 M alkali. Preserve in ceresin-lined bottle.
2. **Sodium Chloride, 5.7% Aqueous.** Dissolve 5.7 gm sodium chloride (NaCl—Merck, Special for Biological Work) in distilled water and dilute to 100 ml.
3. **b-Amylose Cotton (Andersch).** Thoroughly wash 100 gm absorbent cotton (Johnson & Johnson, U.S.P.) three times with distilled water; after the final washing squeeze out the excess fluid and place the cotton in a 2000 ml beaker; pour 1000 ml of 1% starch solution (a) over the cotton; allow to stand for about 4 hours, gently stirring the cotton occasionally to thoroughly saturate it with the starch; transfer the cotton to a wide-stemmed funnel and wash with cold running tap water until the washings, when tested with iodine, show no trace of starch; squeeze out the water; pour over the cotton 250 ml absolute methyl alcohol (CH_3OH—Merck, A.C.S.) making certain that the alcohol thoroughly permeates the cotton; squeeze out the alcohol and add another 250 ml portion in the same manner; squeeze as dry as possible and complete the drying with the aid of an electric fan. Preserve in a tightly capped brown bottle. The b-amylose is stable and the cotton retains its activity for long periods.
   (a) Starch solution, 1% aqueous; in a 1500 ml beaker place 900 ml distilled water and heat to boiling; in the meantime triturate 10 gm soluble starch ($C_6H_{10}O_5$—Merck, Reagent according to Lintner) in a mortar with 50 ml cold water to form a thin smooth paste; slowly and with constant stirring add the contents of the mortar to the boiling water at such a rate that boiling does does not cease; wash out the mortar with another 50 ml portion of water; boil for 1–2 minutes; allow to stand overnight until sediment has settled; decant the clear supernatant fluid for use.

4. **Barium Hydroxide, Volumetric 0.3 N.** Place 600 ml distilled water in a 1000 ml Florence flask and boil for 5 minutes; add 45 gm crystalline barium hydroxide ($Ba(OH)_2 \cdot 8H_2O$—J. T. Baker, A.C.S.) and continue boiling until completely dissolved; immediately fit the flask with a 2-hole rubber stopper carrying syphon tubes, to each of which is attached a soda lime tube; allow to stand until cold—the strength is approximately 0.3 N; syphon off a small amount and titrate against 0.1 N oxalic acid, using phenolphthalein as indicator; calculate the amount necessary to be diluted to 20 ml (or the quantity used in one day) with freshly boiled CO_2-free distilled water to make the strength exactly 0.3 N. Every day, or just before use, make up the volume required—do not dilute the entire stock solution at one time. The solution must be clear and contain no suspended barium carbonate.
5. **Zinc Sulfate, 5% Aqueous.** Dissolve 50 gm crystalline zinc sulfate ($ZnSO_4 \cdot 7H_2O$—J. T. Baker, A.C.S.) in distilled water and dilute to 1000 ml. Titrate against the standardized 0.3 N barium hydroxide (4) with phenolphthalein as follows: place exactly 10 ml of the zinc sulfate in a titration flask and add approximately 100 ml freshly boiled distilled water; add the barium hydroxide, dropwise and with constant agitation, until a pink color persists for at least one minute—10 ml of the sulfate must require 10 ml of the hydroxide—if not, correct accordingly.
6. **Copper-Phosphate-Tartrate Reagent, High Alkalinity (Somogyi).** In a 1500 ml beaker place 700 ml distilled water; add 28 gm anhydrous dibasic sodium phosphate (Na_2HPO_4—Mallinckrodt, A.R.) and stir until dissolved; add 40 gm crystalline sodium potassium tartrate ($NaKC_4H_4O_6 \cdot 4H_2O$—Baker & Adamson, A.C.S.) and stir until dissolved; add 100 ml volumetric 1 N sodium hydroxide (a) and mix; add 80 ml 10% copper sulfate (b) with constant stirring; add 180 gm anhydrous sodium sulfate (Na_2SO_4—Baker & Adamson, Reagent Special); stir until solution is complete; transfer to a 1000 ml mixing cylinder and dilute to volume with distilled water; allow to stand for 2 days until any sediment has settled; filter through Whatman #4 paper. Preserve in a resistant-glass bottle. This reagent is stable indefinitely and shows no deposition of cuprous oxide.
   (a) Sodium hydroxide, volumetric 1 N; prepare by diluting slightly more than the required amount of concentrated carbonate-free sodium hydroxide solution (see Appendix) and standardize against 1 N acid using phenolphthalein as indicator. Preserve in ceresin-lined bottle.
   (b) Copper sulfate, 10% aqueous; dissolve 10 gm finely crystalline copper sulfate ($CuSO_4 \cdot 5H_2O$—Baker & Adamson, A.C.S., Fine Crystals) in distilled water; dilute to 100 ml and filter.
7. **Arsenomolybdate Color Reagent (Nelson).** In a 1000 ml Florence flask place 25 gm ammonium molybdate ($(NH_4)_6Mo_7O_{24} \cdot 4H_2O$—Mallinckrodt, A.R.) and dissolve in 450 ml distilled water; add 21 ml concentrated sulfuric acid (H_2SO_4—Grasselli, A.C.S.) and mix; dissolve 3 gm crystalline sodium acid arsenate ($Na_2HAsO_4 \cdot 7H_2O$—Baker & Adamson, Reagent) in 25 ml distilled water and add to the contents of the flask; mix and place in incubator at 37° C. for 48 hours. Preserve in a brown glass-stoppered bottle.

Technic

Note: For concentrations of total reducing substances in excess of 200 mg per 100 ml in the non-incubated sample, use 0.5 ml sample and 5.5 ml distilled water in Step 10 and multiply the values obtained from the calibration table by 2.

1. In a 125 ml Erlenmeyer flask labelled "A" incubated sample) place
   2 ml phosphate buffer solution (1)
   0.5 ml 5.7% sodium chloride (2)
   0.5 ml sample from an Ostwald-Folin pipette, thoroughly rinsing out the pipette by drawing in and expelling some of the mixture several times.
2. In a similar Erlenmeyer flask labelled "B" (substrate blank) place
   2 ml phosphate buffer solution (1)
   0.5 ml 5.7% sodium chloride (2)
   0.5 ml distilled water.
3. Place both flasks in a water bath at 37.5° C. (± 0.5°) for 5 minutes.
4. To each flask add approximately 1 gm b-amylose cotton (3) and stir with a glass rod until all the fluid is absorbed.
5. Stopper the flasks and replace in the water bath (or incubator) at 37.5° C. for 15 minutes.
6. Add 1 ml 0.3 N barium hydroxide (4) and stir.
7. Add 15 ml distilled water and stir.
8. Add 1 ml 5% zinc sulfate (5).
9. Stir the mixtures thoroughly; decant the supernatant fluids into correspondingly marked centrifuge tubes.
10. In a third 15 ml centrifuge tube labelled "C" (non-incubated sample) place
    5 ml distilled water
    2 ml 0.3 N barium hydroxide (4)
    2 ml 5% zinc sulfate (5)
    1 ml sample from an Ostwald-Folin pipette, added drop by drop with constant agitation of the tube.
11. Centrifuge all tubes at 2000 rpm for 10 minutes.
12. Transfer 2 ml of each clear supernatant centrifugate to a correspondingly marked Folin sugar tube, placing the tip of the pipette near the bottom of the tube so that the fluid does not flow down the walls.

13. In a fourth Folin sugar tube labelled "D" (reagent blank) place 2 ml distilled water.
14. To each tube add 2 ml copper-phosphate-tartrate reagent (6)—the level of the fluid should now fall within the constricted portion of the tube.
15. Mix by lateral shaking and place the tubes in actively boiling water for 10 minutes (the level of the fluid in the tubes must remain below the level of the water in the bath).
16. Cool under running tap water for 3 minutes.
17. Add 2 ml arsenomolybdate color reagent (7), allowing the reagent to flow rapidly down the side of the tube and mix by vigorous lateral shaking.
18. Allow to stand at room temperature for 2 minutes.
19. Dilute to 25 ml with distilled water and mix thoroughly by inverting the tubes three times, allowing the bulbs to empty each time.
20. Measure the transmittancy of the incubated sample "A" against the substrate blank "B" set at 100% transmission at a wave length of **500 mμ** and multiply the value from the calibration table by 4 to obtain the concentration of total reducing substances in mg per 100 ml incubated sample. Record as result (a).
21. Measure the transmittancy of the non-incubated sample "C" against the reagent blank "D" set at 100% transmission at a wave length of **500 mμ** and obtain the concentration of total reducing substances in mg per 100 ml non-incubated sample from the calibration table. Record as result (b).

Calculation

Total reducing substances after incubation (result (a)) in mg per 100 ml − Total reducing substances prior to incubation (result (b)) in mg per 100 ml = Mg reducing substances produced in 100 ml sample expressed as amylase units

CALIBRATION PROCEDURE

Special Apparatus

Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.

Folin sugar tubes, accurately graduated at the 25 ml level.

Reagents

6. **Copper-Phosphate-Tartrate Reagent, High Alkalinity (Somogyi).** Same as for Test Procedure.
7. **Arsenomolybdate Color Reagent (Nelson).** Same as for Test Procedure.
8. **Benzoic Acid, 0.25% Aqueous.** In a 1500 ml beaker heat 1000 ml distilled water to boiling; add 2.5 gm benzoic acid ($C_7H_6O_2$—Baker & Adamson, Reagent) and stir until dissolved; allow to cool; adjust volume to 1000 ml with distilled water and filter.
9. **Dextrose Standards**
   (a) Dextrose stock standard (1 ml contains 10 mg dextrose). Dry dextrose ($C_6H_{12}O_6$—National Bureau of Standards) over sulfuric acid in vacuo to constant weight; transfer 1 gm to a 100 ml volumetric flask; add 50 ml 0.25% benzoic acid (8); shake until dissolved; dilute to volume with same and mix. Stability indefinite.
   (b) Dilute standard (1 ml contains 2 mg dextrose). In a 100 ml volumetric flask place 20 ml stock standard (9a) and dilute to volume with 0.25% benzoic acid (8); mix. Stability approximately one month in refrigerator.
   (c) Calibration standards. In a series of five accurately calibrated 100 ml volumetric flasks place respectively 0, 2.5, 5.0, 7.5 and 10 ml of the dilute standard (9b) and dilute to volume with distilled water. These standards represent concentrations of dextrose equivalent to 0, 50, 100, 150 and 200 mg per 100 ml sample. Prepare just before use and proceed immediately as follows.

Technic

1. Transfer 2 ml of each calibration standard (9c) to a correspondingly marked Folin sugar tube.
2. Proceed exactly as outlined in the Test Procedure, Steps 14–19 inclusive.
3. Set the zero concentration standard at 100% transmission at a wave length of **500 mμ** and record the transmittancy of the 50, 100, 150 and 200 mg per cent standards. The transmittancy of the 200 mg standard should be approximately 19%.
4. Plot the observed values on semi-log graph paper—the points fall on a straight line which passes through the origin (Calibration Curve A-35).
5. Prepare a calibration table from the T-C values obtained from the plotted curve.

PROCEDURE FOR USE WITH LINEAR-SCALE PHOTOELECTRIC COLORIMETERS (SHEARD-SANFORD TYPE)

TEST PROCEDURE

Exactly as outlined; set the blanks at 100 and use a green filter.

CALIBRATION PROCEDURE

Exactly as outlined—use a green filter; plot the observed readings on semi-log graph paper—the points fall on a straight line passing through the origin.

PROCEDURE FOR USE WITH LOGARITHMIC-SCALE PHOTOELECTRIC COLORIMETERS (KLETT-SUMMERSON TYPE)

TEST PROCEDURE

Exactly as outlined; set the blanks at 0 and use a green filter.

CALIBRATION PROCEDURE

Exactly as outlined—use a green filter; plot the observed readings on linear graph paper—the points fall on a straight line passing through the origin.

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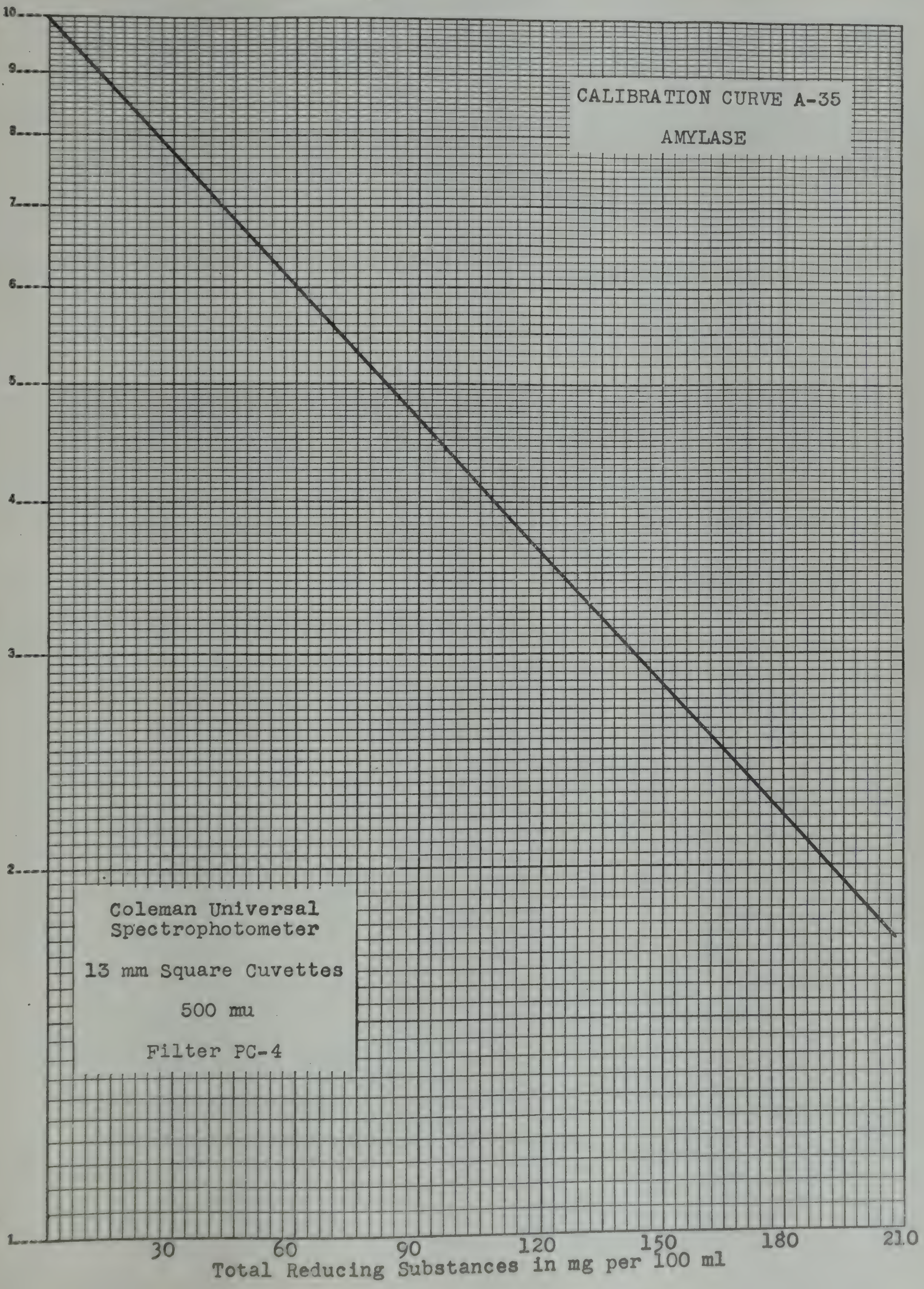
NOTES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AMYLASE
Coleman Universal Spectrophotometer
13 mm Square Cuvettes
500 mu
Filter PC-4
10
9
8
7
6
5
4
3
2
1
30
60
90
120
150
180
210
Total Reducing Substances in mg per 100 ml

Meter Reading	0	1	2	3	4	5	6	7	8	9
0										
10										
20										
30										
40										
50										
60										
70										
80										
90										

AMYLASE

A spectrophotometric adaptation based on the methods and modifications of Myers, Free and Rosinski (1944), Swanson (1948) and Smith and Roe (1949)

PRINCIPLE

Amylase produces a blue color with starch and iodine. If the density of the blue color is measured before and after incubation, the decrease in intensity due to the hydrolysis of the starch is proportional to the concentration of amylase. One amylase unit is defined as the amount of enzyme that will hydrolyze 10 mg starch (in the presence of 60 mg) in 30 minutes to a stage at which no color is given under the conditions of the test.

DETERMINATION IN PLASMA OR SERUM

SAMPLE

Plasma or serum from blood withdrawn at any time of the day but preferably at the height of an attack—analyses preferably made on serum. Anticoagulant for plasma—potassium oxalate. Carry out protein precipitation as soon as possible, although amylase activity, if high, is not significantly decreased in samples refrigerated overnight.

TEST PROCEDURE

Special Apparatus

Ostwald-Folin pipettes accurately calibrated "to deliver" 1 ml.

Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.

Reagents

1. **Starch Substrate (Smith-Roe).** Just prior to use place exactly 1.2 gm soluble starch ($C_6H_{10}O_5$—Merck, according to Lintner) in a 100 ml Pyrex volumetric flask and add 10 ml distilled water; shake until evenly suspended and then dilute to slightly less than 100 ml with boiling distilled water; immerse the flask in boiling water for 3 minutes and dilute to volume with boiling distilled water; replace flask in water bath, allow to cool to 90° C. and maintain at this temperature while using. Discard excess.
2. **Phosphate Buffer Solution, 0.2 M, pH 7.2 (Myers-Free-Rosinski).** In a 1000 ml volumetric flask place 7.62 gm monobasic potassium phosphate (KH_2PO_4—Merck, Soerensen) and 20.45 gm anhydrous dibasic sodium phosphate (Na_2HPO_4—Merck, Special for pH); add approximately 800 ml distilled water; shake until dissolved and dilute to volume. Preserve in resistant-glass bottle.
3. **Sodium Chloride, 0.5 M.** Dissolve 29.227 gm sodium chloride (NaCl—Merck, Special for Biological Work) in distilled water and dilute to 1000 ml.
4. **Hydrochloric Acid, Volumetric 1 N.** Add 100 ml concentrated hydrochloric acid (HCl—Baker & Adamson, A.C.S.) to 1000 ml distilled water and mix; standardize against 1 N alkali using phenolphthalein as indicator.
5. **Iodine Reagent (Smith-Roe, Modified).** In a 1000 ml volumetric flask place 15 gm potassium iodide (KI—J. T. Baker, Low-Iodate, Special); add 20 ml distilled water and shake until dissolved; add 15 gm resublimed iodine (I—Baker & Adamson, A.C.S.); shake until dissolved and dilute to volume with distilled water.

Technic

Note: For concentrations of amylase in excess of 500 units per 100 ml, dilute the plasma or serum with an equal volume of 0.85% sodium chloride before proceeding with Step 5 and multiply the final result by 2.

1. In an 18 × 150 mm test tube labelled "A" (digested sample) place
   5 ml hot starch substrate (1)
   3 ml phosphate buffer solution (2) and mix
   1 ml 0.5 M sodium chloride (3) and mix.
2. In a second 18 × 150 mm test tube labelled "B" (non-digested sample) place
   5 ml hot starch substrate (1)
   3 ml phosphate buffer solution (2) and mix
   1 ml 0.5 M sodium chloride (3) and mix.
3. In a third 18 × 150 mm test tube labelled "C" (sample blank) place
   5 ml distilled water
   3 ml phosphate buffer solution (2) and mix
   1 ml 0.5 M sodium chloride (3) and mix.
4. Place the tubes in a water bath at 37° C. and allow to remain until the contents of the tubes have reached the temperature of the bath (5–10 minutes).
5. Remove tube "A" from the water bath, add 1 ml plasma or serum from an Ostwald-Folin pipette, added drop by drop with constant agitation of the tube and mix by gentle rotatory motion, replace in bath with the other tubes.
6. Allow all tubes to remain in the bath at 37° C. for exactly 30 minutes.
7. Remove all tubes from the bath and immediately add 2 ml 1 N hydrochloric acid (4) to each tube and mix thoroughly.
8. Add 1 ml plasma or serum to tubes "B" and "C" in the same manner as before and mix thoroughly.
9. In each of three correspondingly marked 250 ml volumetric flasks place about 200 ml distilled water, 2.5 ml 1 N hydrochloric acid (4) and mix.

10. Transfer 1 ml from each test tube to the appropriate flask and mix.
11. To each flask add 1 ml iodine reagent (5) and mix thoroughly.
12. Dilute to volume with distilled water.
13. Measure the optical density (red scale) of the digested sample "A" against the sample blank "C" set at 0 density at a wave length of 620 mμ. Record as result (a).
14. Measure the optical density (red scale) of the non-digested sample "B" against the sample blank "C" set at 0 density at a wave length of 620 mμ. Record as result (b).
15. Calculate the concentration of amylase as follows.

Calculation

$$\frac{\text{Optical density of non-digested sample (result (b))} - \text{Optical density of digested sample (result (a))}}{\text{Optical density of non-digested sample (result (b))}} \times 600 = \text{Amylase units per 100 ml plasma or serum}$$

CALIBRATION PROCEDURE

A calibration curve cannot be used with this method. Due to variations in the starch substrate and the temperature, the density of the digested sample must always be compared with the density of the non-digested sample using the same starch preparation.

Method A-36b

DETERMINATION IN URINE

SAMPLE

Aliquot of a 24-hour specimen or individual specimens as indicated. Keep cool during collection.

TEST PROCEDURE

Special Apparatus

As listed under the Test Procedure for blood.

Reagents

1. **Starch Substrate (Smith-Roe).** Same as for blood.
2. **Phosphate Buffer Solution, 0.2 M, pH 7.2 (Myers-Free-Rosinski).** Same as for blood.
3. **Sodium Chloride, 0.5 M.** Same as for blood.
4. **Hydrochloric Acid, Volumetric 1 N.** Same as for blood.
5. **Iodine Reagent (Smith-Roe, Modified).** Same as for blood.

Technic

Note: For concentrations of amylase in excess of 500 units per 100 ml, dilute the urine with an equal volume of distilled water before proceeding with Step 2 and multiply the final result by 2.

1. Filter a small portion of thoroughly mixed urine.
2. Proceed exactly as outlined in the Test Procedure for blood, Steps 1–15 inclusive.

Calculation

Same as for blood.

PROCEDURE FOR USE WITH LINEAR-SCALE PHOTOELECTRIC COLORIMETERS (SHEARD-SANFORD TYPE)

TEST PROCEDURE

Exactly as outlined; set the sample blank at 100 and use a filter with maximum transmission at 620 mμ. Obtain the equivalent densities from the Transmission-Optical Density Chart (see Appendix, Chart X-1) and calculate as outlined.

PROCEDURE FOR USE WITH LOGARITHMIC-SCALE PHOTOELECTRIC COLORIMETERS (KLETT-SUMMERSON TYPE)

TEST PROCEDURE

Exactly as outlined; set the sample blank at 0 and use a filter with maximum transmission at 620 mμ. Since the logarithmic scale readings bear a definite relationship to the density, calculations may be made directly.

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AMYLASE

A spectrophotometric adaptation based on the methods and modifications of Sherman, Kendall and Clark (1910), Myers and Killian (1917), Somogyi (1931; 1937; 1938), Shaffer and Somogyi (1933) and Myers, Free and Rosinski (1944)

PRINCIPLE

Plasma or serum is incubated for a definite period with a starch substrate, buffered at pH 7.2, in the presence of optimum sodium chloride concentration. The concentration of total reducing substances before and after digestion is determined by means of an alkaline copper-phosphomolybdate reaction as for total reducing substances (Method D-10), the same analytical principles being involved. The difference between these two values (the reducing substances produced per 100 ml of enzyme solution in 30 minutes) is considered as the measure of the amylolytic power of the sample. The results are expressed in terms of amylase units, one unit being equivalent to 1 mg total reducing substances per 100 ml sample.

DETERMINATION IN PLASMA OR SERUM

SAMPLE

Plasma or serum from blood withdrawn at any time of the day but preferably at the height of an attack—analyses preferably made on serum. Anticoagulant for plasma—potassium oxalate. Carry out protein precipitation as soon as possible, although amylase activity, if high, is not significantly decreased in samples refrigerated overnight. The same type of sample, that is, either plasma or serum, must be used for the determination of total reducing substances before and after digestion.

TEST PROCEDURE

Special Apparatus

Ostwald-Folin pipettes accurately calibrated "to deliver" 1 ml.

Folin sugar tubes—the 4 ml level must fall within the constricted portion of the tube and the 25 ml graduation must be accurately calibrated. (For a more detailed description see Method D-10.)

Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.

Reagents

1. **Sodium Chloride, 0.5% Aqueous.** Dissolve 5 gm sodium chloride (NaCl—Merck, Special for Biological Work) in distilled water and dilute to 1000 ml.
2. **Starch Substrate, 1.5% (Somogyi).** In a 250 ml beaker place 90 ml distilled water and heat to boiling; in the meantime place 1.5 gm purified starch powder (a) in a small glass mortar and triturate with 2 ml distilled water to form a thin paste; when the water boils decant the starch paste into the beaker with constant stirring; wash out the mortar with 8 ml distilled water, transferring the washings to the beaker; boil for 1 minute, stirring constantly; transfer the beaker to a water bath at 40° C. and allow to remain for 30 minutes, keeping the beaker covered during this period; transfer the contents of the beaker to a 100 ml mixing cylinder and adjust to volume with distilled water; mix by inversion. The solution may be preserved by transferring, while still hot, to a sterile bottle and sealing with a rubber cap (similar to that used for vaccine bottles)—for use, the required amount may be withdrawn aseptically. Preserve in refrigerator and discard when the starch settles out—stability approximately one month.
    - (a) Starch powder, purified (Somogyi); in a liter mixing cylinder place 1000 ml 0.01 N hydrochloric acid (i) and add 100 gm corn starch ($C_6H_{10}O_5$—J. T. Baker, U.S.P.); agitate vigorously and frequently for one hour; allow the suspended starch to settle and carefully decant and discard the supernatant fluid; add 1000 ml 0.05% sodium chloride (ii) and again agitate as before for one hour; allow to settle, decant and discard the supernatant fluid; repeat the washing with sodium chloride; filter through a Büchner funnel and suck as dry as possible; spread the starch on clean white paper and allow to dry in air, keeping well protected from dust.
        - (i) Hydrochloric acid, 0.01 N; dilute 1 ml concentrated hydrochloric acid (HCl—Baker & Adamson, A.C.S.) to 1000 ml with distilled water and mix; this solution need not be standardized.
        - (ii) Sodium chloride, 0.05% aqueous; dilute 100 ml 0.5% sodium chloride (1) to 1000 ml with distilled water.
3. **Phosphate Buffer Solution, 0.2 M, pH 7.2 (Myers-Free-Rosinski).** In a 1000 ml volumetric flask place 7.62 gm monobasic potassium phosphate (KH_2PO_4—Merck, Soerensen) and 20.45 gm anhydrous dibasic sodium phosphate (Na_2HPO_4—Merck, Special for pH); add approximately 800 ml distilled water; shake until dissolved and dilute to volume. Preserve in resistant-glass bottle.
4. **Sodium Chloride, 0.5 M.** Dissolve 29.227 gm sodium chloride (NaCl—Merck, Special for Biological Work) in distilled water and dilute to 1000 ml.

5. **Copper Sulfate, 5% Aqueous.** Dissolve 5 gm finely crystalline copper sulfate ($CuSO_4 \cdot 5H_2O$—Baker & Adamson, A.C.S., Fine Crystals) in distilled water; dilute to 100 ml and filter.
6. **Sodium Tungstate, 6% Aqueous.** Dissolve 6 gm sodium tungstate ($Na_2WO_4 \cdot 2H_2O$—Mallinckrodt, Folin Special) in distilled water and dilute to 100 ml. This solution must be neutral or slightly alkaline to phenolphthalein, not more than 0.24 ml being required to neutralize 10 ml of 0.1 N hydrochloric acid. Preserve in resistant-glass bottle.
7. **Copper Reagent, High Alkalinity (Shaffer-Somogyi, Modified).** In a 1000 ml volumetric flask place 700 ml distilled water; add 25 gm anhydrous sodium carbonate (Na_2CO_3—Baker & Adamson, A.C.S.) and shake until dissolved; add 25 gm sodium potassium tartrate ($NaKC_4H_4O_6 \cdot 4H_2O$—Baker & Adamson, A.C.S.) and shake until dissolved; add 40 ml 1 N sodium hydroxide (a) and mix thoroughly; add 60 ml 10% copper sulfate (b) and mix thoroughly; dilute to volume with distilled water, mix and filter through a Whatman #4 paper. Preserve in resistant-glass bottle.
    - (a) Sodium hydroxide, volumetric 1 N; prepare by diluting slightly more than the required amount of concentrated carbonate-free sodium hydroxide solution (see Appendix) and standardize against 1 N acid using phenolphthalein as indicator. Preserve in ceresin-lined bottle.
    - (b) Copper sulfate, 10% aqueous; dissolve 10 gm finely crystalline copper sulfate ($CuSO_4 \cdot 5H_2O$—Baker & Adamson, A.C.S., Fine Crystals) in distilled water; dilute to a 100 ml and filter.
8. **Phosphomolybdic Acid Reagent, Concentrated (Folin Improved).** In a 1000 ml Florence flask place 500 ml clear, supernatant, brominated sodium molybdate solution (a); add 225 ml 85% phosphoric acid (H_3PO_4—Baker & Adamson, A.C.S.)—the solution becomes yellow due to liberated bromine; add 150 ml cool 25% sulfuric acid (b); aerate until free of bromine as evidenced by a colorless solution (usually in 10–15 minutes); transfer to a 1000 ml volumetric flask; add 75 ml glacial acetic acid (CH_3COOH—Baker & Adamson, A.C.S.); dilute to volume with distilled water and mix thoroughly. Stability indefinite.
    - (a) Sodium molybdate, brominated; in a 1000 ml volumetric flask place 700 ml distilled water; add 300 gm sodium molybdate ($Na_2MoO_4 \cdot 2H_2O$—Mallinckrodt, AR) and shake until dissolved; add 2–3 drops bromine (Br—Baker & Adamson, A.C.S.); dilute to volume with distilled water and mix. On long standing a precipitate may form which does not affect the usefulness of the solution.
    - (b) Sulfuric acid, 25% aqueous; dilute one volume concentrated sulfuric acid (H_2SO_4—Grasselli, A.C.S.) with three volumes distilled water; allow to cool before use.
9. **Phosphomolybdic Acid Reagent, Dilute.** In a 100 ml graduated mixing cylinder place 20 ml concentrated phosphomolybdic acid reagent (8) and dilute to volume with distilled water; mix thoroughly. Prepare fresh daily.

Technic

Note: When the amylolytic power is greater than 400 amylase units, dilute the sample with 0.5% sodium chloride (1) so that the amount of total reducing substances is less than 400 mg per 100 ml before proceeding with Step 4 (a 1:2 or 1:3 dilution is usually sufficient); make the appropriate correction for the dilution used when obtaining the results from the calibration table.

1. In each of two 18 × 150 mm Pyrex test tubes labelled "A" and "B" place
   5 ml starch substrate (2)
   3 ml phosphate buffer solution (3) and mix
   1 ml 0.5 M sodium chloride (4) and mix.
2. In a third 18 × 150 mm pyrex test tube labelled "C" place
   5 ml distilled water
   3 ml phosphate buffer solution (3) and mix
   1 ml 0.5 M sodium chloride (4) and mix.
3. Place the tubes in a water bath at 40° C. and allow to remain until the contents of the tubes have reached the temperature of the bath (5–10 minutes).
4. From an Ostwald-Folin pipette, add drop by drop with constant agitation of the tube, 1 ml sample to the tubes marked "A" (sample + substrate) and "C" (sample + water).
5. Add 1 ml distilled water to the tube marked "B" (substrate + water).
6. Place the tubes in a water bath at 40° C. for exactly 30 minutes.
7. Remove the tubes from the water bath at the end of exactly 30 minutes and immediately add 1 ml 5% copper sulfate (5) to each, starting with tube "A", and mix.

Note: Steps 7–10 inclusive must be carried out without undue delay; addition of the copper sulfate inhibits but does not halt the action of the enzyme.

8. Add 1 ml 6% sodium tungstate (6) and shake vigorously until foam-free.
9. Add 8 ml distilled water and mix.
10. Filter the contents of each tube through a Whatman #4 paper into a correspondingly marked test tube, keeping the funnels covered during filtration—the filtrates should show no more than a slight opalescence.
11. Transfer 2 ml of each filtrate to a correspondingly marked Folin sugar tube, placing the tip of the pipette near the bottom of the tube so that the fluid does not flow down the walls.

12. In a fourth Folin sugar tube labelled "D" (reagent blank) place 2 ml distilled water.
13. To each tube add 2 ml copper reagent (7)—the level of the fluid should now fall within the constricted portion of the tube.
14. Mix by lateral shaking and place the tubes in actively boiling water for 20 minutes (the level of the fluid in the tubes must remain below the level of the water in the bath).
15. Cool under running tap water for 3 minutes.
16. Add 4 ml concentrated phosphomolybdic acid reagent (8), allowing the reagent to flow rapidly down the side of the tube and mix by vigorous lateral shaking.
17. Replace in boiling water for exactly 5 minutes.
18. Cool as before for 3 minutes.
19. Dilute to the 25 ml level with *dilute* phosphomolybdic acid reagent (9).
20. Mix thoroughly by inverting the tubes three times, allowing the bulbs to empty each time.
21. Allow to stand at room temperature for 2 minutes —if the solutions are not crystal clear, transfer portions to 15 ml centrifuge tubes and centrifuge at 1500 rpm for 3 minutes.
22. Measure the transmittancy of tubes "A", "B" and "C" against tube "D" set at 100% transmission at a wave length of 500 mμ and obtain the concentration of total reducing substances in mg per 100 ml from the calibration table.
23. Calculate the amylase content as follows.

Calculation

Total reducing substances in tube "A" (reducing substances in serum and starch + reducing substances formed by the amylase) — Total reducing substances in tube "B" (reducing substances in starch) — Total reducing substances in tube "C" (reducing substances in serum) = Mg reducing substances produced in 100 ml sample expressed as amylase units.

CALIBRATION PROCEDURE

Special Apparatus

Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.

Folin sugar tubes, accurately graduated at the 25 ml level.

Reagents

7. **Copper Reagent, High Alkalinity (Shaffer-Somogyi, Modified).** Same as for Test Procedure.

8. **Phosphomolybdic Acid Reagent, Concentrated (Folin Improved).** Same as for Test Procedure.

9. **Phosphomolybdic Acid Reagent, Dilute.** Same as for Test Procedure.

10. **Benzoic Acid, 0.25% Aqueous.** In a 1500 ml beaker heat 1000 ml distilled water to boiling; add 2.5 gm benzoic acid ($C_7H_6O_2$—Baker & Adamson, Reagent) and stir until dissolved; allow to cool; adjust volume to 1000 ml with distilled water and filter.

11. **Dextrose Standards**
    (a) Dextrose stock standard (1 ml contains 10 mg dextrose). Dry dextrose ($C_6H_{12}O_6$—National Bureau of Standards) over sulfuric acid in vacuo to constant weight; transfer 1 gm to a 100 ml volumetric flask; add 50 ml 0.25% benzoic acid (10); shake until dissolved; dilute to volume with same and mix. Stability indefinite.
    (b) Dilute standard (1 ml contains 2 mg dextrose). In a 100 ml volumetric flask place 20 ml stock standard (11a); dilute to volume with 0.25% benzoic acid (10) and mix. Stable for approximately one month in refrigerator.
    (c) Calibration standards. In a series of five accurately calibrated 100 ml volumetric flasks place respectively 0, 2.5, 5, 7.5 and 10 ml of the dilute standard (11b) and dilute to volume with distilled water; mix. These standards represent concentrations of dextrose equivalent to 0, 100, 200, 300 and 400 mg per 100 ml sample. Prepare just before use.

Technic

1. Transfer 2 ml of each calibration standard (11c) to a correspondingly marked Folin sugar tube.

2. Proceed exactly as outlined in the Test Procedure, Steps 13–21 inclusive.

3. Set the zero concentration standard at 100% transmission at a wave length of 500 mμ and record the transmittancy of the 100, 200, 300 and 400 mg per cent standards. The transmittancy of the 400 mg standard should be approximately 26%.

4. Plot the observed values on semi-log graph paper —the points fall on a straight line which passes through the origin (Calibration Curve A-37).

5. Prepare a calibration table from the T-C values obtained from the plotted curve.

Method A-37b

DETERMINATION IN WHOLE BLOOD

The procedure is carried out exactly as outlined for plasma or serum except that 7% copper sulfate (12) and 10% sodium tungstate (13) are used instead of 5% copper sulfate and 6% sodium tungstate.

Reagents

12. **Copper Sulfate, 7% Aqueous.** Dissolve 7 gm finely crystalline copper sulfate ($CuSO_4 \cdot 5H_2O$—Baker & Adamson, A.C.S., Fine Crystals) in distilled water; dilute to 100 ml and filter.
13. **Sodium Tungstate, 10% Aqueous.** Dissolve 10 gm sodium tungstate ($Na_2WO_4 \cdot 2H_2O$—Mallinckrodt, Folin Special) in distilled water and dilute to 100 ml. The solution must be neutral or slightly alkaline to phenolphthalein, not more than 0.4 ml being required to neutralize 10 ml 0.1 N hydrochloric acid. Preserve in resistant-glass bottle.

PROCEDURE FOR USE WITH LINEAR-SCALE PHOTOELECTRIC COLORIMETERS (SHEARD-SANFORD TYPE)

TEST PROCEDURE

Exactly as outlined; set the reagent blank at 100 and use a blue filter.

CALIBRATION PROCEDURE

Exactly as outlined—use a blue filter; plot the observed readings on semi-log graph paper—the points fall on a straight line passing through the origin.

PROCEDURE FOR USE WITH LOGARITHMIC-SCALE PHOTOELECTRIC COLORIMETERS (KLETT-SUMMERSON TYPE)

TEST PROCEDURE

Exactly as outlined; set the reagent blank at 0 and use a blue filter.

CALIBRATION PROCEDURE

Exactly as outlined—use a blue filter; plot the observed readings on linear graph paper—the points fall on a straight line passing through the origin.

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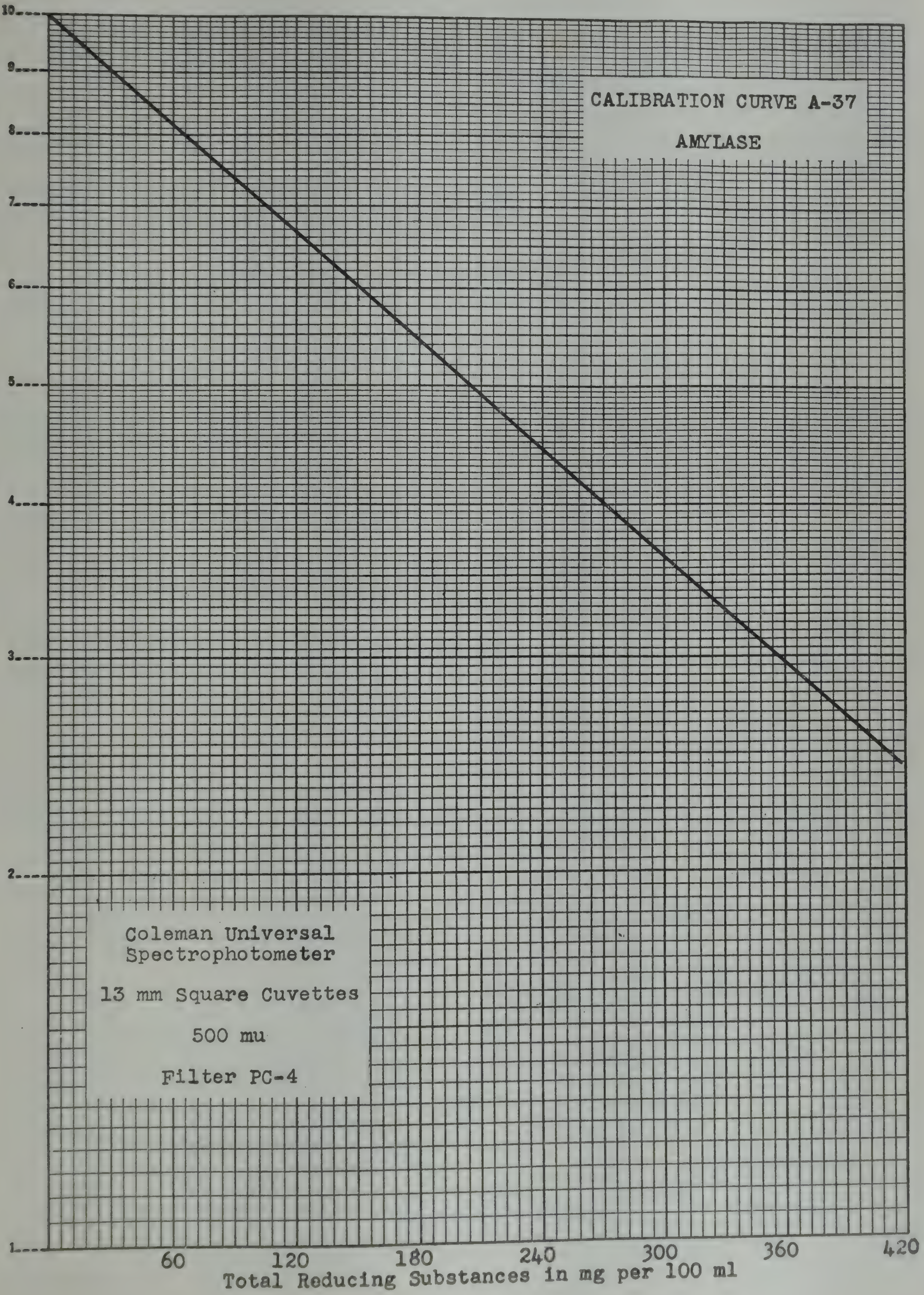
NOTES


CALIBRATION CURVE A-37
AMYLASE
Coleman Universal Spectrophotometer
13 mm Square Cuvettes
500 mu
Filter PC-4
10
9
8
7
6
5
4
3
2
1
60
120
180
240
300
360
420
Total Reducing Substances in mg per 100 ml

Meter Reading	0	1	2	3	4	5	6	7	8	9
0										
10										
20										
30										
40										
50										
60										
70										
80										
90										

ASCORBIC ACID

A spectrophotometric adaptation based on the methods and modifications of Emmerie and Van Eekelen (1934), Pijoan and Klemperer (1937), Mindlin and Butler (1938), Bukatsch (1939), Stotz (1941) and Palmer (1941)

PRINCIPLE

Ascorbic acid is determined by means of its reducing action on the dye, 2,6-dichlorobenzenone indophenol (Tillmans reaction). To a solution of the dye (which is blue in alkaline solution and red in acid solution) is added an amount of protein-free metaphosphoric acid filtrate of plasma or serum, insufficient to produce complete reduction. The unreduced fraction of dichlorobenzenone indophenol is determined after extraction with xylene, thus indicating the amount of dye reduced and indirectly the amount of ascorbic acid.

DETERMINATION IN PLASMA OR SERUM

SAMPLE

Plasma or serum from blood withdrawn during postabsorptive state. Anticoagulant—potassium oxalate in combination with potassium cyanide as a preservative (Reagent #1). Precautions must be taken to avoid any trace of hemolysis (i.e., dry syringes and needles and gentle shaking). The determination is best carried out within 30 minutes from the time blood is collected. The plasma or serum should not be separated from the red cells until just before the analysis is started. Filtrates may be kept in the refrigerator for several hours without appreciably diminishing the ascorbic acid value.

TEST PROCEDURE

Special Apparatus

Ostwald-Folin pipettes accurately calibrated "to contain" 3 ml.
Erlenmeyer flasks, glass-stoppered, 50 ml.
Capillary droppers delivering 40 drops per ml.
Rapid-delivery pipettes, 2 ml and 12 ml.
Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.

Reagents

1. **Oxalate-Cyanide Mixture.** In the tubes to be used for the collection of the blood place 0.1 ml (15 mg) 15% potassium oxalate (a) and 0.1 ml (10 mg) 10% potassium cyanide (b); allow to evaporate at room temperature or in a drying oven at 90° C.—unless the mixture in the tubes is dry, hemolysis may occur. This amount of mixture is adequate for preventing the coagulation of 10 ml of blood.
    (a) Potassium oxalate, 15% aqueous; dissolve 15 gm crystalline potassium oxalate ($K_2C_2O_4 \cdot H_2O$—J. T. Baker, A.C.S.) in distilled water and dilute to 100 ml.
    (b) Potassium cyanide, 10% aqueous; dissolve 10 gm potassium cyanide (KCN—Baker & Adamson, Reagent) in distilled water and dilute to 100 ml. Prepare frequently in small amounts.
2. **Water, Distilled, Carbon Dioxide-Free.** In a 500 ml flask place 300 ml distilled water; boil for 5 minutes; insert rubber stopper with soda-lime guard and allow to cool to room temperature. This must be used for the preparation of all reagents and standards and where indicated in the test procedure.
3. **Metaphosphoric Acid, 6% Aqueous.** Using only clear pellets, dissolve 6 gm glacial metaphosphoric acid (HPO_3—Baker & Adamson, Reagent) in carbon dioxide-free distilled water (2) and dilute to 100 ml. Although this reagent remains stable for 1–2 weeks in the refrigerator it is better to prepare small amounts fresh each day.
4. **Bromcresol Green Indicator Solution, 0.04% Aqueous (Clark).** In a 250 ml volumetric flask place 102 mg of the water-soluble sodium salt of bromcresol green ($C_{21}H_{14}Br_4O_5S$—LaMotte); dissolve in carbon dioxide-free distilled water (2) and dilute to volume. Preserve in resistant-glass bottle.
5. **Sodium Hydroxide, Volumetric 0.8 N.** Prepare by diluting slightly more than the required amount of concentrated carbonate-free sodium hydroxide solution (see Appendix) and titrate against standard 1 N acid using phenolphthalein as indicator; adjust accordingly and re-titrate—20 ml of 1 N acid require 25 ml of 0.8 N alkali. Preserve in ceresin-lined bottle.
6. **Phosphate-Citrate Buffer Solution, pH 4.0.** Mix 30 ml 0.2 M disodium phosphate (a) and 50 ml 0.2 M citric acid (b). Check pH potentiometrically and adjust, if necessary, with more of either phosphate or citric acid. Add a few drops of xylene (8) and preserve in resistant-glass bottle.
    (a) Sodium phosphate, dibasic, 0.2 M; dry powdered dibasic sodium phosphate (Na_2HPO_4—Merck, Special for pH) at 110° C. for one hour; transfer 28.396 gm to a 1000 ml volumetric flask; add about 800 ml carbon dioxide-free distilled water (2) and shake until dissolved; dilute to volume. Preserve in resistant-glass bottle.
    (b) Citric acid, 0.2 M; in a 1000 ml volumetric flask place 42.016 gm powdered recrystallized citric acid ($H_3C_6H_5O_7 \cdot H_2O$—Reagent Chemicals, Special); add about 800 ml carbon dioxide-free distilled water (2) and

shake until dissolved; dilute to volume. Preserve in resistant-glass bottle.

7. **2,6-Dichlorobenzenone Indophenol, 0.02% Aqueous.** Dissolve 20 mg sodium 2,6-dichlorobenzenone indophenol ($NaOC_6H_4N:C_6H_2(Cl_2):O$—Eastman #3463) in 75 ml boiling distilled water; cool, filter and adjust volume to 100 ml with carbon dioxide-free distilled water (2). Always filter before use. Stability 8–10 days in refrigerator.
8. **Xylene** ($C_6H_4(CH_3)_2$—Baker & Adamson, A.C.S.).

Technic

1. In a 15 ml centrifuge tube place
   3 ml carbon dioxide-free distilled water (2)
   3 ml sample from an Ostwald-Folin pipette, thoroughly rinsing out the pipette by drawing in and expelling some of the mixture several times.
2. Add 6 ml 6% metaphosphoric acid (3) and mix thoroughly by vigorous shaking for 30 seconds.
3. Allow to stand for 15 minutes.
4. Centrifuge at 2500 rpm for 15 minutes (or filter through a dry 10 cm Whatman #40 paper into a dry tube).
5. Transfer 8 ml clear supernatant fluid (or filtrate) to a 50 ml glass-stoppered Erlenmeyer flask (sample).
6. In another 50 ml glass-stoppered Erlenmeyer flask (reagent blank) place
   4 ml carbon dioxide-free distilled water (2)
   4 ml 6% metaphosphoric acid (3).
7. To each flask add 1 drop bromcresol green indicator solution (4) from a capillary-tipped dropper.
8. Add from a capillary-tipped dropper, dropwise and with constant shaking, just enough 0.8 N sodium hydroxide (5) to produce a green color.
9. Add 1 ml phosphate-citrate buffer solution (6) and mix.
10. Using a rapid-delivery pipette, add 2 ml 2,6-dichlorobenzenone indophenol solution (7).
11. Rotate the flask for thorough mixing and within 30 seconds after the addition of the dye solution add exactly 12 ml xylene (8) from a rapid-delivery pipette.
12. Stopper the flasks and shake vigorously for 15 seconds; the xylene layer in both flasks must be tinted pink—if the layer in the flask containing the sample is not pink add another 1 ml of the dye solution (7) to *each* flask and shake again.
13. Allow the layers to separate for a few minutes and then carefully decant the xylene layers through dry 7 cm Whatman #40 papers and dry funnels into dry cuvettes—the color in the xylene layers is stable for hours.
14. Measure the transmittancy of the sample against the reagent blank set at *50%* transmission at a wave length of **500 mμ** and obtain the concentration of ascorbic acid from the calibration table.

CALIBRATION PROCEDURE

Special Apparatus

Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.
Erlenmeyer flasks, glass-stoppered, 50 ml.
Capillary droppers delivering 40 drops per ml.
Rapid-delivery pipettes, 2 ml and 12 ml.

Reagents

2. **Water, Distilled, Carbon Dioxide-Free.** Same as for Test Procedure.
3. **Metaphosphoric Acid, 6% Aqueous.** Same as for Test Procedure.
4. **Bromcresol Green Indicator Solution, 0.04% Aqueous (Clark).** Same as for Test Procedure.
5. **Sodium Hydroxide, Volumetric 0.8 N.** Same as for Test Procedure.
6. **Phosphate-Citrate Buffer Solution, pH 4.0.** Same as for Test Procedure.
7. **2,6-Dichlorobenzenone Indophenol, 0.02% Aqueous.** Same as for Test Procedure.
8. **Xylene.** Same as for Test Procedure.
9. **Metaphosphoric Acid-Cyanide Solution.** In a 100 ml volumetric flask place 0.25 ml 10% potassium cyanide (1b) and dilute to volume with 6% metaphosphoric acid (3). Prepare just before use.
10. **Ascorbic Acid Standards**
    (a) Ascorbic acid stock standard (1 ml contains 1 mg ascorbic acid). In a 100 ml volumetric flask place 100 mg crystalline ascorbic acid ($C_6H_8O_6$—U.S.P. Reference Standard); add about 75 ml carbon dioxide-free distilled water (2) and shake until dissolved; dilute to volume. Prepare not more than one hour before use. Note: The concentration may be checked by titrating 10 ml with 0.01 N iodine to a faint permanent blue color with 1 drop 0.5% starch indicator solution—1 ml 0.01 N iodine is equivalent to 0.877 mg ascorbic acid.
    (b) Dilute standard (1 ml contains 0.0125 mg ascorbic acid). In a 200 ml volumetric flask place 2.5 ml stock standard (10a) and dilute to volume with carbon dioxide-free distilled water (2). Prepare just before use.
    (c) Calibration standards. In a series of six accurately calibrated 10 ml volumetric flasks place respectively 0, 1, 2, 3, 4 and 5 ml of the dilute standard (10b); add 5, 4, 3, 2, 1 and 0 ml respectively carbon dioxide-free distilled water (2); add 5 ml metaphosphoric acid-cyanide solution (9) and

mix. These standards represent concentrations of ascorbic acid equivalent to 0, 0.5, 1.0, 1.5, 2.0 and 2.5 mg per 100 ml sample. Use immediately after preparation.

Technic

1. Transfer 8 ml of each calibration standard (10c) to a correspondingly marked 50 ml glass-stoppered Erlenmeyer flask.
2. Proceed exactly as outlined in the Test Procedure, Steps 7–13 inclusive.
3. Set the zero concentration standard at 50% transmission at a wave length of 500 mμ and record the transmittancy of the 0.5, 1.0, 1.5, 2.0 and 2.5 mg per cent standards. The transmittancy of the 2.5 mg standard should be approximately 63%.
4. Plot the observed values on semi-log graph paper —the points fall on a straight line which intersects the transmission axis at 50% and slopes upward (Calibration Curve A-45).
5. Prepare a calibration table from the T-C values obtained from the plotted curve.

Method A-45b

DETERMINATION IN URINE

Note: Although the results obtained by dichlorobenzenone indophenol procedures for the determination of ascorbic acid in urine are influenced by other substances present in the urine, the following technic is included here because of its widespread use. For very accurate work the method of Roe and Kuether (Method A-46b) should be used.

PRINCIPLE

Interfering substances in the urine are removed by the addition of barium acetate and the ascorbic acid determined as outlined for blood.

SAMPLE

Aliquot of a 24-hour specimen—concentrations in individual voidings vary greatly. Collect in amber bottles fitted with plastic screw caps (do not use corks or metal caps) containing as a preservative sufficient solid metaphosphoric acid to produce a final concentration of 3%—this may be accomplished by adjusting approximately after the final voiding or by placing 30 gm of the solid acid in quart bottles before collection is started and diluting bottles containing less than 1000 ml to 1 liter with distilled water; in either case, the total volume voided must be known and due correction made in the calculation of the final result. Keep in refrigerator during collection and until analysis can be carried out.

TEST PROCEDURE

Special Apparatus

Mixing cylinders, 10 ml, accurately calibrated.
Erlenmeyer flasks, glass-stoppered, 50 ml.
Capillary droppers delivering 40 drops per ml.
Rapid-delivery pipettes, 2 ml and 12 ml.
Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.

Reagents

2. **Water, Distilled, Carbon Dioxide-Free.** Same as for blood.
3. **Metaphosphoric Acid, 6% Aqueous.** Same as for blood.
4. **Bromcresol Green Indicator Solution, 0.04% Aqueous (Clark).** Same as for blood.
5. **Sodium Hydroxide, Volumetric 0.8 N.** Same as for blood.
6. **Phosphate-Citrate Buffer Solution, pH 4.0.** Same as for blood.
7. **2,6-Dichlorobenzenone Indophenol, 0.02% Aqueous.** Same as for blood.
8. **Xylene.** Same as for blood.
11. **Barium Acetate, Powder.** ($Ba(C_2H_3O_2)_2 \cdot H_2O$ —Reagent Chemicals, Special.)
12. **Metaphosphoric Acid, 3% Aqueous.** Dilute 50 ml 6% metaphosphoric acid (3) with an equal volume of carbon dioxide-free distilled water (2). Prepare fresh daily.

Technic

Note: For concentrations of ascorbic acid in excess of 2.5 mg per 100 ml, use less centrifugate in Step 5 and sufficient 3% metaphosphoric acid (12) to bring the volume to 10 ml; for concentrations less than 0.5 mg per 100 ml, use more centrifugate, maintaining the final volume at 10 ml; correct the values obtained from the calibration table for blood accordingly.

1. Filter a portion of thoroughly mixed urine.
2. In a 15 ml centrifuge tube place
   10 ml filtered urine
   100 mg barium acetate powder (11).
3. Insert a clean rubber stopper and shake for 5 minutes.
4. Centrifuge at 1800 rpm for 5 minutes.
5. Transfer 2.5 ml clear supernatant centrifugate to an accurately calibrated 10 ml mixing cylinder.
6. Dilute to 10 ml with 3% metaphosphoric acid (12) and mix.
7. Transfer 8 ml diluted urine to a 50 ml glass-stoppered Erlenmeyer flask (sample).
8. Proceed exactly as outlined in the Test Procedure for blood, Steps 6–13 inclusive.
9. Measure the transmittancy of the sample against

the reagent blank set at *50%* transmission at a wave length of 500 mμ; obtain the value from the calibration table for blood and calculate the concentration of ascorbic acid as follows.

Calculation

$$\text{Value obtained from calibration table} \times \frac{\text{Total volume of diluted urine}}{\text{Actual volume of 24-hour specimen}} = \text{Concentration of ascorbic acid in mg per 100 ml urine}$$

$$\frac{\text{Volume of 24-hour specimen}}{100} \times \text{Ascorbic acid in mg per 100 ml} = \text{Mg ascorbic acid excreted in 24 hours}$$

PROCEDURE FOR USE WITH LINEAR-SCALE PHOTOELECTRIC COLORIMETERS (SHEARD-SANFORD TYPE)

TEST PROCEDURE

Exactly as outlined; set the reagent blank at 50 and use a green filter.

CALIBRATION PROCEDURE

Exactly as outlined—use a green filter; plot the observed readings on semi-log graph paper—the points fall on a straight line which intersects the transmission axis at 50% and slopes upward.

PROCEDURE FOR USE WITH LOGARITHMIC-SCALE PHOTOELECTRIC COLORIMETERS (KLETT-SUMMERSON TYPE)

TEST PROCEDURE

Exactly as outlined; set the reagent blank at 150 and use a green filter.

CALIBRATION PROCEDURE

Exactly as outlined—use a green filter; plot the observed readings on linear graph paper—the points fall on a straight line which intersects the instrument reading axis at 150 and slopes downward.

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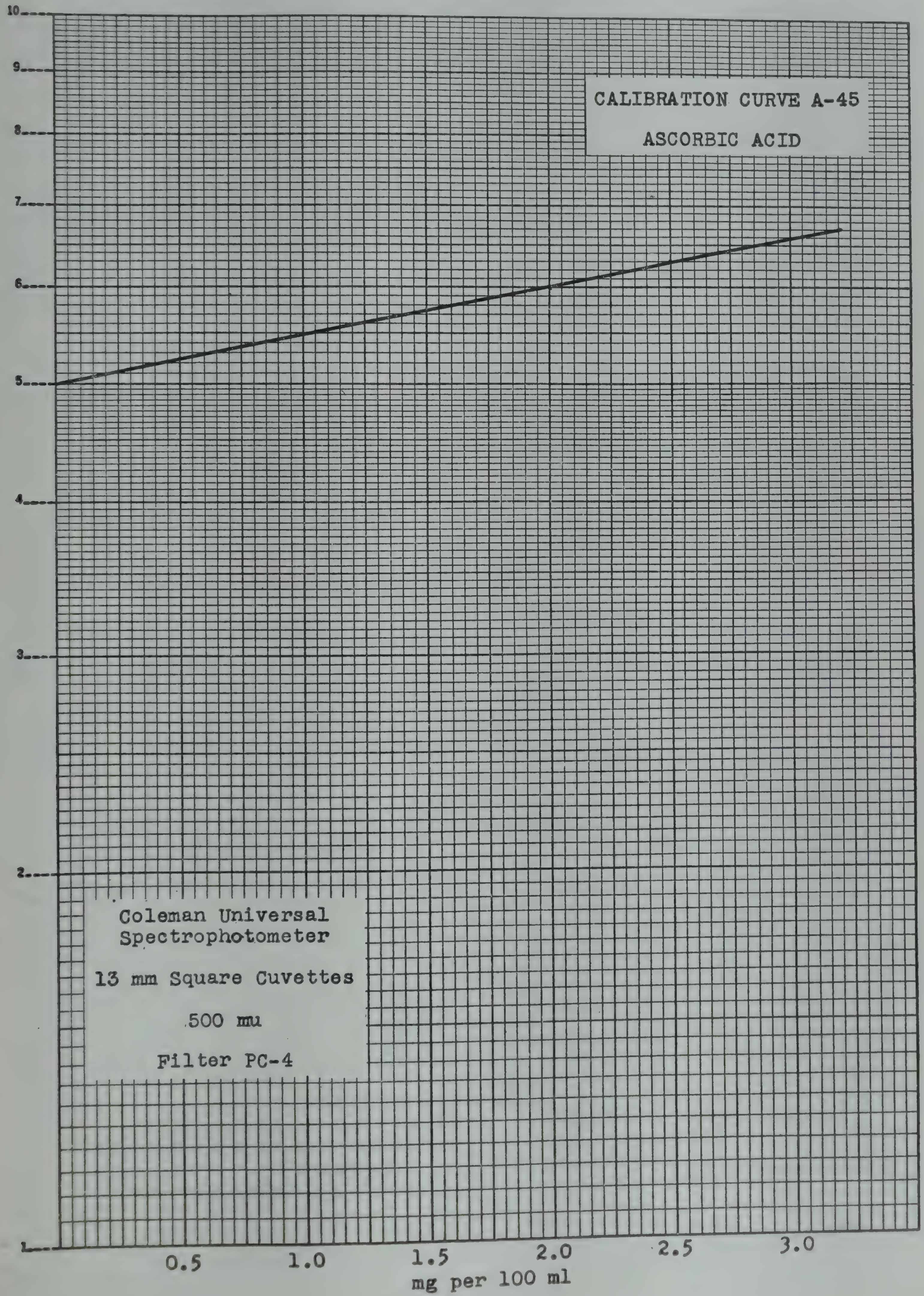
CALIBRATION CURVE A-45
ASCORBIC ACID
10
9
8
7
6
5
4
3
2
1
Coleman Universal
Spectrophotometer
13 mm Square Cuvettes
500 mu
Filter PC-4
0.5
1.0
1.5
2.0
2.5
3.0
mg per 100 ml

Meter Reading	0	1	2	3	4	5	6	7	8	9
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10										
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80										
90										

ASCORBIC ACID

A spectrophotometric adaptation based on the methods and modifications of Roe and Kuether (1942; 1943), Lowry, Lopez and Bessey (1945) and Mills and Roe (1947)

PRINCIPLE

Ascorbic acid, in a protein-free trichloracetic acid filtrate, is oxidized by means of norit to dehydroascorbic acid which is then coupled with 2,4-dinitrophenylhydrazine and the resulting derivative treated with sulfuric acid to produce a reddish color. Thiourea is used to prevent interference by other oxidants.

DETERMINATION IN WHOLE BLOOD, PLASMA OR SERUM

SAMPLE

Blood withdrawn during post-absorptive state. Anticoagulant—potassium oxalate. There must be no trace of hemolysis. Whole blood analyses are preferable. The trichloracetic acid filtrate may be kept for several days in the refrigerator without affecting the ascorbic acid value.

TEST PROCEDURE

Special Apparatus

Centrifuge tubes, glass-stoppered, 50 ml.
Ostwald-Folin pipettes accurately calibrated "to deliver" 5 ml.
Filter paper, Schleicher and Schuell #576, 9 cm.
Graduated test tubes—25 ml in 0.5 ml.
Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.

Reagents

1. **Norit Suspension (Lowry-Lopez-Bessey).** In a 100 ml mixing cylinder place 5 gm acid-washed norit A (a) and add 5% trichloracetic acid (b) to the 100 ml mark; shake thoroughly, allow to settle, decant and discard the supernatant fluid and restore to volume with 5% trichloracetic acid; repeat this washing process several times until the majority of the fine floating charcoal is removed and finally dilute to a volume of 100 ml with 5% trichloracetic acid. Decant and discard the supernatant fluid weekly and restore to volume with 5% trichloracetic acid.
   (a) Norit A, acid-washed (Roe-Kuether); some samples of norit A are unsatisfactory for use without further treatment. To determine whether additional washing is necessary, run a blank test on trichloracetic acid which has been passed through the carbon to be used—if no more color is produced with trichloracetic acid thus treated than with untreated trichloracetic acid, further treatment of the carbon is unnecessary. If a high blank is obtained, treat the norit A as follows—in a 2000 ml beaker place 200 gm norit A (Pfanstiehl) and add 1000 ml 10% hydrochloric acid (by volume); heat to boiling and filter with suction; transfer the norit to another 2000 ml beaker and add 1000 ml distilled water; mix thoroughly and filter. Repeat this procedure until the washings give a negative or very faint test for ferric iron. Dry in hot air oven at 110° C. for 24 hours—do not heat to high temperatures.
   (b) Trichloracetic acid, 5% aqueous; dilute 5 ml concentrated trichloracetic acid (Reagent #4a) to 100 ml with distilled water. Prepare fresh weekly.
2. **Dinitrophenylhydrazine Reagent (Lowry-Lopez-Bessey).** In a 100 ml mixing cylinder place 2 gm 2,4-dinitrophenylhydrazine ($(NO_2)_2$-C_6H_3 $NHNH_2$—Eastman #1866) and make up to volume with 9 N sulfuric acid (a); shake until dissolved; add 250 mg thiourea (NH_2CSNH_2—Eastman #497) and again shake until solution is complete; if an opalescence or precipitate develops, centrifuge or filter through a sintered glass funnel. Stability one month.
   (a) Sulfuric acid, volumetric 9 N; slowly add 300 ml concentrated sulfuric acid (H_2SO_4—Grasselli, A.C.S.) to 750 ml distilled water; mix well and allow to cool; dilute 10 ml of this approximately 10 N solution to 100 ml; titrate against standard 1 N alkali using phenolphthalein as indicator; adjust accordingly and re-titrate—20 ml of a 1:10 dilution of 10 N acid require 20 ml of 1 N alkali. To prepare 9 N acid dilute 90 ml of the 10 N acid to 100 ml with distilled water; allow to cool and readjust volume. Preserve in Pyrex glass-stoppered bottle.
3. **Sulfuric Acid, 65% Aqueous.** In a 1500 ml Florence flask place 300 ml distilled water; while constantly shaking the flask under running tap water, add slowly 700 ml concentrated sulfuric acid (H_2SO_4—Grasselli, A.C.S.); allow to cool and dilute to 1000 ml with distilled water. Preserve in Pyrex glass-stoppered bottle.

Technic

1. In a 50 ml glass-stoppered centrifuge tube place
   15 ml norit suspension (1)
   5 ml sample from an Ostwald-Folin pipette,

added drop by drop, with constant agitation of the tube.

2. Stopper the tube and shake vigorously for 2 minutes.
3. Allow to stand for 10 minutes.
4. Centrifuge at 1500 rpm for 5 minutes.
5. Decant the supernatant fluid through a dry 9 cm Schleicher and Schuell #576 paper into a dry test tube—the filtrate must show no trace of carbon; refilter if necessary.
6. Into each of two 25 ml graduated test tubes place 4 ml clear filtrate.
7. To one tube (sample) add 1 ml dinitrophenylhydrazine reagent (2) and mix; reserve the other tube until Step 9.
8. Place tube in a constant temperature water bath at 37° C. for 3 hours.
9. Remove tube from the water bath and place in an ice water bath containing generous quantities of ice, together with the other tube (sample blank) reserved in Step 7.
10. To each tube slowly add 7 ml ice-cold 65% sulfuric acid (3) from a 10 ml burette, agitating the tubes in the ice bath during the addition of the acid.

Note: Do not use stop-cock grease on the burette—lubricate with sulfuric acid. It is essential that during the addition of the acid, the temperature of the solution does not rise appreciably.

11. Add 1 ml dinitrophenylhydrazine reagent (2) to the sample blank tube.
12. Agitate both tubes thoroughly while still in the ice water bath until the contents are completely mixed.
13. Remove tubes from ice bath and allow to stand at room temperature for 30 minutes.
14. Measure the transmittancy of the sample against the sample blank set at 100% transmission at a wave length of **520** mμ and obtain the concentration of ascorbic acid from the calibration table.

CALIBRATION PROCEDURE

Special Apparatus

Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.
Centrifuge tubes, glass-stoppered, 50 ml.
Graduated test tubes—25 ml in 0.5 ml.

Reagents

1. **Norit Suspension (Lowry-Lopez-Bessey).** Same as for Test Procedure.
2. **Dinitrophenylhydrazine Reagent (Lowry-Lopez-Bessey).** Same as for Test Procedure.
3. **Sulfuric Acid, 65% Aqueous.** Same as for Test Procedure.
4. **Trichloracetic Acid, 4% Aqueous.** Dilute 4 ml concentrated trichloracetic acid (a) to 100 ml with distilled water. Prepare fresh weekly.
   (a) Trichloracetic acid, concentrated; dissolve 100 gm crystalline trichloracetic acid (CCl_3-COOH—J. T. Baker, CP Analyzed) in distilled water and dilute to 100 ml. Preserve in Pyrex glass-stoppered bottle. Stability indefinite.
5. **Ascorbic Acid Standards**
   (a) Ascorbic acid stock standard (1 ml contains 1 mg ascorbic acid). In a 100 ml volumetric flask place 100 mg crystalline ascorbic acid ($C_6H_8O_6$—U.S.P. Reference Standard); add approximately 50 ml 4% trichloracetic acid (4) and shake until dissolved; dilute to volume with 4% trichloracetic acid. Prepare shortly before use. Note: the concentration may be checked by titrating 10 ml with 0.01 N iodine to a faint permanent blue color with 1 drop 0.5% starch indicator solution—1 ml 0.01 N iodine is equivalent to 0.877 mg ascorbic acid.
   (b) Dilute standard (1 ml contains 0.075 mg ascorbic acid). In a 100 ml volumetric flask place 7.5 ml stock standard (5a) and dilute to volume with 4% trichloracetic acid (4). Prepare immediately before use.
   (c) Calibration standards. In a series of five 50 ml glass-stoppered centrifuge tubes place respectively 0, 1, 2, 3 and 4 ml of the dilute standard (5b); add 20, 19, 18, 17 and 16 ml norit suspension (1) respectively, bringing the volume in each tube to 20 ml. These standards represent concentrations of ascorbic acid equivalent to 0, 1.5, 3.0, 4.5 and 6.0 mg per 100 ml sample. Proceed immediately as follows.

Technic

1. Stopper the tubes containing the calibration standards (5c) and shake vigorously for 2 minutes.
2. Allow to stand for 10 minutes.
3. Centrifuge at 1500 rpm for 5 minutes.
4. Decant the supernatant fluids through dry 9 cm Schleicher and Schuell #576 papers into dry correspondingly marked test tubes—the filtrates must show no trace of carbon; refilter if necessary.
5. Transfer 4 ml of the clear filtrates to correspondingly marked 25 ml graduated test tubes.
6. To each tube add 1 ml dinitrophenylhydrazine reagent (2) and mix.
7. Place the tubes in a constant temperature water bath at 37° C. for 3 hours.
8. Transfer the tubes to an ice water bath containing generous quantities of ice.
9. To each tube slowly add 7 ml ice-cold 65% sulfuric acid (3) from a 10 ml burette, agitating the tubes in the ice bath during the addition of the acid.

Note: Do not use stop-cock grease on the burette—lubricate with sulfuric acid. It is essential that during the addition of the acid, the temperature of the solution does not rise appreciably.

10. Agitate the tubes thoroughly while still in the ice water bath until the contents are completely mixed.
11. Remove from ice bath and allow to stand at room temperature for 30 minutes.
12. Set the zero concentration standard at 100% transmission at a wave length of **520** mμ and record the transmittancy of the 1.5, 3.0, 4.5 and 6.0 mg per cent standards. The transmittancy of the 6 mg standard should be approximately 24%.
13. Plot the observed values on semi-log graph paper—the points fall on a smooth curve which passes through the origin (Calibration Curve A-46).
14. Prepare a calibration table from the T-C values obtained from the plotted curve.

Method A-46b

DETERMINATION IN URINE

SAMPLE

Aliquot of a 24-hour specimen. Keep cold during collection.

TEST PROCEDURE

Special Apparatus

Centrifuge tubes, glass-stoppered, 50 ml.
Ostwald-Folin pipettes accurately calibrated "to deliver" 1 ml.
Graduated test tubes—25 ml in 0.5 ml.
Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.

Reagents

1. **Norit Suspension (Lowry-Lopez-Bessey).** Same as for blood.
2. **Dinitrophenylhydrazine Reagent (Lowry-Lopez-Bessey).** Same as for blood.
3. **Sulfuric Acid, 65% Aqueous.** Same as for blood.

Technic

Note: For concentrations of ascorbic acid in excess of 30 mg per 100 ml, use 1 ml urine and 39 ml norit suspension in Step 2 and multiply the values obtained from the calibration table for blood by 10.

1. Filter a small portion of well mixed urine.
2. In a 50 ml glass-stoppered centrifuge tube place
   19 ml norit suspension (1)
   1 ml filtered urine from an Ostwald-Folin pipette, added drop by drop, with constant agitation of the tube.
3. Stopper the tube and shake vigorously for 2 minutes.
4. Proceed exactly as outlined in the Test Procedure for blood, Steps 3–13 inclusive.
5. Measure the transmittancy of the sample against the sample blank set at 100% transmission at a wave length of **520** mμ and multiply the value from the calibration table for blood by 5 to obtain the concentration of ascorbic acid in mg per 100 ml urine.

Calculation

$$\frac{\text{Volume of 24-hour specimen}}{100} \times \text{Ascorbic acid in mg per 100 ml} = \text{Mg ascorbic acid excreted in 24 hours}$$

PROCEDURE FOR USE WITH LINEAR-SCALE PHOTOELECTRIC COLORIMETERS (SHEARD-SANFORD TYPE)

TEST PROCEDURE

Exactly as outlined; set the sample blank at 100 and use a green filter.

CALIBRATION PROCEDURE

Exactly as outlined—use a green filter; plot the observed readings on semi-log graph paper—the points fall on a slight curve passing through the origin.

PROCEDURE FOR USE WITH LOGARITHMIC-SCALE PHOTOELECTRIC COLORIMETERS (KLETT-SUMMERSON TYPE)

TEST PROCEDURE

Exactly as outlined; set the sample blank at 0 and use a green filter.

CALIBRATION PROCEDURE

Exactly as outlined—use a green filter; plot the observed readings on linear graph paper—the points fall on a slight curve passing through the origin.

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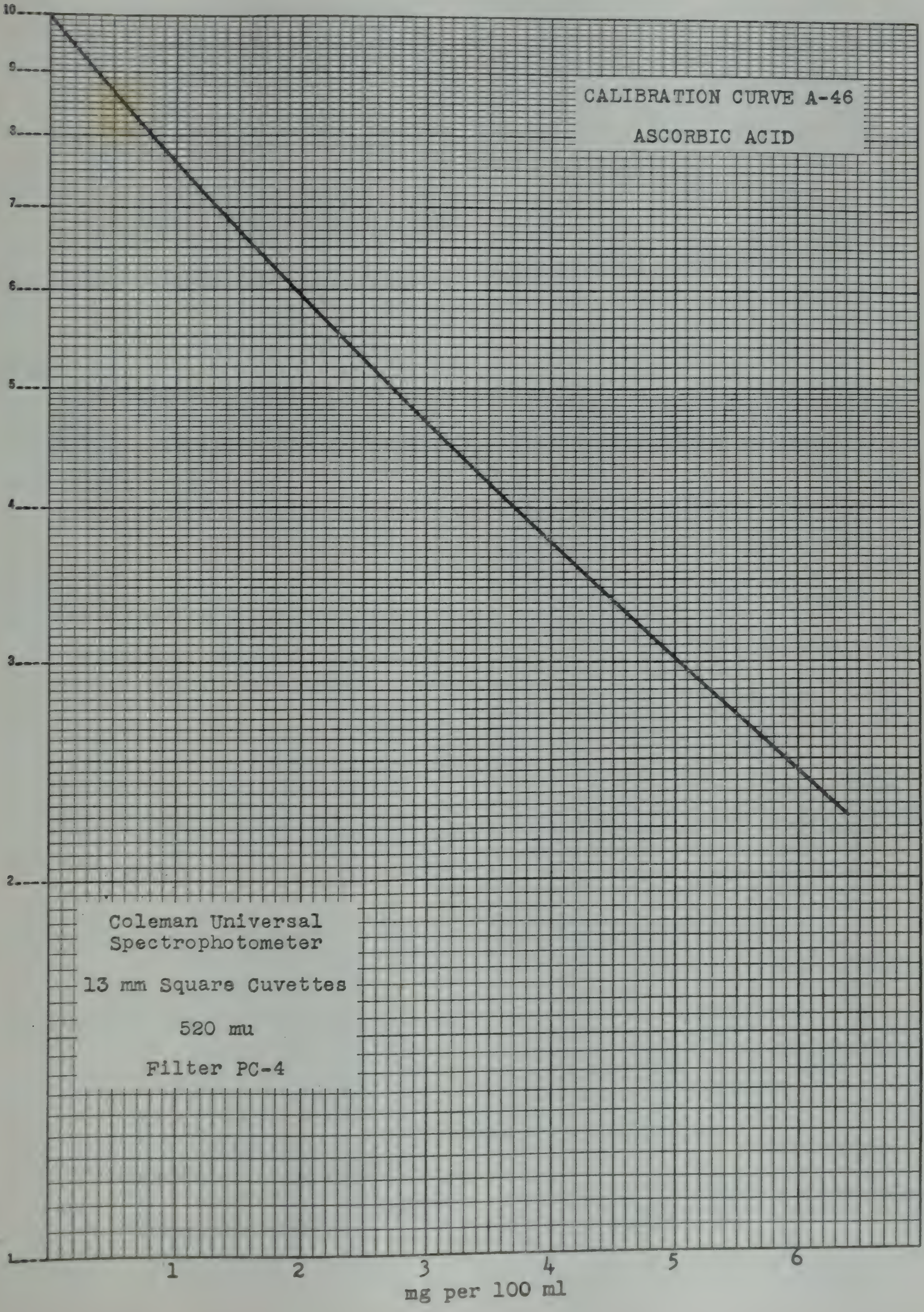
NOTES


CALIBRATION CURVE A-46
ASCORBIC ACID
10
9
8
7
6
5
4
3
2
1
Coleman Universal
Spectrophotometer
13 mm Square Cuvettes
520 mu
Filter PC-4
1
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mg per 100 ml

Meter Reading	0	1	2	3	4	5	6	7	8	9
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BARBITURIC ACID

A spectrophotometric adaptation based on the methods and modifications of Koppanyi, Murphy and Krop (1933), Koppanyi, Dille, Murphy and Krop (1934), Dille and Koppanyi (1934), Kozelka and Tatum (1937), Brundage and Gruber (1937), Delmonico (1939), Cohen (1946) and Bacila and Alcaide (1948)

PRINCIPLE

The acidified urine is evaporated to dryness and the residue extracted with chloroform. After removal of pigments by means of charcoal, the extract is evaporated to dryness, taken up in alcohol and treated with cobalt nitrate and ammonia in the presence of glycerine to produce a reddish-purple color. This method is non-specific, neither very sensitive nor accurate and unsuitable for the determination of certain derivatives of barbituric acid. It is included here only because of its widespread use. The best recovery possible is approximately 85%. For precise work, ultraviolet fluorescence methods should be used.

DETERMINATION IN URINE

SAMPLE

Aliquot of a 24-hour specimen. Single samples frequently supply the necessary information—in the case of unstable derivatives they are essential. Sulfonamides interfere with the color reaction.

TEST PROCEDURE

Special Apparatus

Graduated test tubes—10 ml in 0.1 ml; the 5 ml graduation must be accurately calibrated.

Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.

Reagents

1. **Sulfuric Acid, 10% Aqueous.** Slowly add 10 ml concentrated sulfuric acid (H_2SO_4—Grasselli, A.C.S.) to 90 ml distilled water and mix; allow to cool and adjust volume to 100 ml.
2. **Chloroform, Anhydrous** ($CHCl_3$—Baker & Adamson, A.C.S.).
3. **Activated Charcoal (Brundage-Gruber).** Suspend 100 gm norit A (Pfanstiehl) in 500 ml distilled water and stir vigorously; allow to settle and decant the supernatant fluid; again suspend in distilled water, allow to settle and decant; suspend in 500 ml 5% hydrochloric acid, allow to settle and decant; dry in hot air oven at 110° C. for 24 hours—do not heat to high temperatures. A small amount of the dried carbon, when suspended in water, should give an acid reaction with litmus.
4. **Methyl Alcohol** (CH_3OH—Merck, A.C.S.).
5. **Glycerine** ($C_3H_5(OH)_3$—Baker & Adamson, A.C.S.).
6. **Cobalt Nitrate, 1.5% Alcoholic.** Dissolve 1.5 gm crystalline cobaltous nitrate ($Co(NO_3)_2 \cdot 6H_2O$—Baker & Adamson, A.C.S.) in methyl alcohol (4) and dilute to 100 ml with same. Preserve in brown glass-stoppered bottle. Discard when a brown color appears.
7. **Ammonium Hydroxide, 3% Alcoholic.** Dilute 3 ml concentrated ammonium hydroxide (NH_4OH—Baker & Adamson, A.C.S.) to 100 ml with methyl alcohol (4).

Technic

Note: For concentrations of barbituric acid less than 1 mg per 100 ml, use a larger sample in Step 1; for concentrations in excess of 20 mg, use a smaller sample. In either case, correct the values obtained from the calibration table accordingly.

1. In a 400 ml Pyrex beaker place 50 ml thoroughly mixed urine.
2. Add sufficient 10% sulfuric acid (1) to render distinctly acid to litmus paper.
3. Evaporate to just dryness on a sand bath.

Note: For the remainder of the procedure the maintenance of anhydrous conditions is essential.

4. Add 20 ml chloroform (2) to the residue and evaporate to half-volume on the sand bath.
5. Transfer the extract through a Schleicher and Schuell #576 paper into a dry 100 ml Pyrex beaker.
6. Repeat Steps 4 and 5 twice, transferring the extracts through the same filter paper into the beaker.
7. Evaporate the combined extracts to a volume of 5 ml on the sand bath.
8. Add 250 mg activated charcoal (3) and mix thoroughly.
9. Transfer to a 30 ml Pyrex beaker through a Schleicher and Schuell #576 paper, rinsing the 100 ml beaker with five 2 ml portions chloroform (2) and transferring the washings through the same paper into the 30 ml beaker.
10. Evaporate the combined washings to just dryness on the sand bath.
11. Add 2 ml methyl alcohol (4) and gently agitate the beaker until solution of the residue is complete.
12. Add 3 drops glycerine (5) and mix by gentle agitation.
13. While constantly agitating the beaker, apply gentle heat until the contents boils momentarily.

14. Quantitatively transfer to a 10 ml graduated test tube (sample), rinsing the beaker with not more than 2.5 ml methyl alcohol (4) and transferring the washings to the tube.
15. In another 10 ml graduated test tube (reagent blank) place 2 ml methyl alcohol (4) and 3 drops glycerine (5); mix and heat to momentary boiling.
16. To each tube add 0.3 ml 1.5% cobalt nitrate (6) and mix.
17. Adjust the volumes to exactly 5 ml with methyl alcohol (4).
18. Add exactly 1 ml 3% ammonium hydroxide (7) and mix.
19. Within 5 minutes, measure the transmittancy of the sample against the reagent blank set at 100% transmission at a wave length of **565** mμ and obtain the concentration of barbituric acid from the calibration table.

CALIBRATION PROCEDURE

Note: The procedure as outlined may be used for the determination of various derivatives of barbituric acid provided the compound which is to be determined is also used in the calibration procedure—it is not possible to employ one barbiturate as a standard for determining other barbiturates. Neither can factors, based on the ratio of the molecular weights, be used since the color response of the various barbiturates is not proportional to their molecular weights.

Special Apparatus

Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.

Graduated test tubes—10 ml in 0.1 ml; the 5 ml graduation must be accurately calibrated.

Reagents

4. **Methyl Alcohol.** Same as for Test Procedure.
5. **Glycerine.** Same as for Test Procedure.
6. **Cobalt Nitrate, 1.5% Alcoholic.** Same as for Test Procedure.
7. **Ammonium Hydroxide, 3% Alcoholic.** Same as for Test Procedure.
8. **Barbituric Acid Standards**
   (a) Barbituric acid stock standard (1 ml contains 5 mg diethyl barbituric acid). In a 100 ml volumetric flask place 500 mg diethyl barbituric acid ($C_4H_4O_3N_2 \cdot 2H_2O$—Merck, U.S.P.); add 80 ml methyl alcohol (4); shake until dissolved and dilute to volume with same. Preserve in glass-stoppered bottle.
   (b) Calibration standards. In a series of five accurately calibrated 10 ml graduated test tubes place respectively 0, 0.5, 1.0, 1.5 and 2.0 ml of the stock standard (8a); add 2.0, 1.5, 1.0, 0.5 and 0 ml methyl alcohol (4) respectively, bringing the volume in each tube to 2 ml. These standards represent concentrations of barbituric acid equivalent to 0, 5, 10, 15 and 20 mg per 100 ml sample. Prepare just before use and proceed immediately as follows.

Technic

1. To each calibration standard (8b) add 3 drops glycerine (5), mix by gentle agitation and heat gently to momentary boiling.
2. Proceed exactly as outlined in the Test Procedure, Steps 16–18 inclusive.
3. Within 5 minutes, set the zero concentration standard at 100% transmission at a wave length of **565** mμ and record the transmittancy of the 5, 10, 15 and 20 mg per cent standards. The transmittancy of the 20 mg standard should be approximately 34%.
4. Plot the observed values on semi-log graph paper—the points fall on a straight line which passes through the concentration axis slightly to the right of the origin (Calibration Curve B-5).
5. Prepare a calibration table from the T-C values obtained from the plotted curve.

PROCEDURE FOR USE WITH LINEAR-SCALE PHOTOELECTRIC COLORIMETERS (SHEARD-SANFORD TYPE)

TEST PROCEDURE

Exactly as outlined; set the reagent blank at 100 and use a green filter.

CALIBRATION PROCEDURE

Exactly as outlined—use a green filter; plot the observed readings on semi-log graph paper—the points fall on a straight line which passes through the concentration axis slightly to the right of the origin.

PROCEDURE FOR USE WITH LOGARITHMIC-SCALE PHOTOELECTRIC COLORIMETERS (KLETT-SUMMERSON TYPE)

TEST PROCEDURE

Exactly as outlined; set the reagent blank at 0 and use a green filter.

CALIBRATION PROCEDURE

Exactly as outlined—use a green filter; plot the observed readings on linear graph paper—the points fall on a straight line which passes through the concentration axis slightly to the right of the origin.

Note: For instruments with cells, cuvettes or tubes requiring a final volume of more than 6 ml, add 2 ml methyl alcohol just before readings are made in both the test and calibration procedures.

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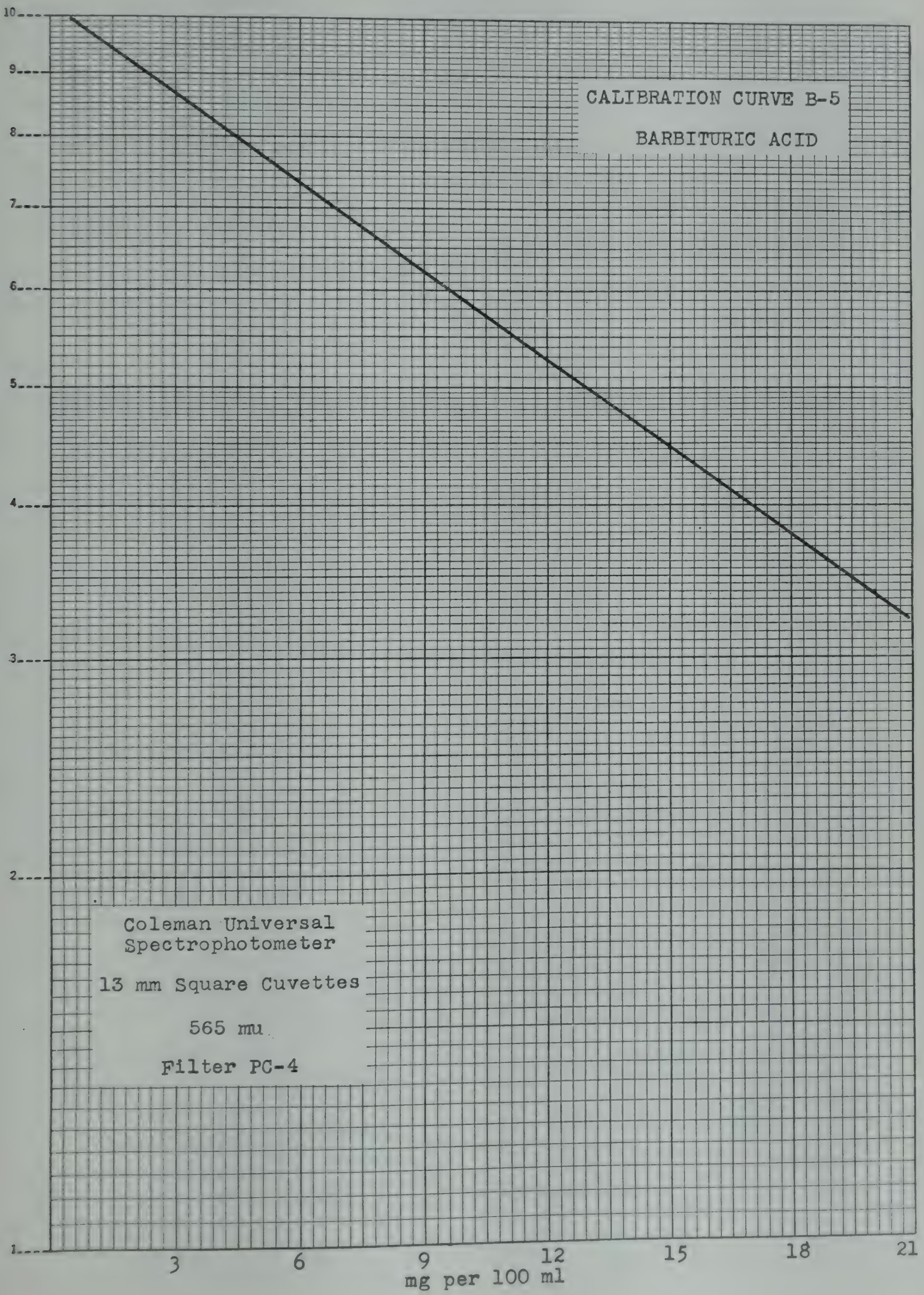
CALIBRATION CURVE B-5
BARBITURIC ACID
Coleman Universal Spectrophotometer
13 mm Square Cuvettes
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Filter PC-4
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mg per 100 ml

Meter Reading	0	1	2	3	4	5	6	7	8	9
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BILIRUBIN

A spectrophotometric adaptation based on the methods and modifications of Van den Bergh and Snapper (1913), Van den Bergh and Muller (1916), Davis and Sheard (1934), Malloy and Evelyn (1937), Hubbard and Heilbrun (1941), Watson (1942) and Ducci and Watson (1945)

PRINCIPLE

Total bilirubin is determined in alcoholic solution by combination with diazotized sulfanilic acid to form red-violet azobilirubin (Ehrlich reaction). Direct-reacting bilirubin is estimated in aqueous solution—the prompt direct fraction by a one minute reading and the total direct fraction by a fifteen minute reading, the difference between these two forms being considered as the delayed direct-reacting bilirubin. The percentage promptness of the direct reaction is the ratio between the one and fifteen minute readings. The amount of indirect bilirubin is the difference between the total direct-reacting bilirubin and the total bilirubin.

DETERMINATION IN PLASMA OR SERUM

SAMPLE

Plasma or serum from blood withdrawn during post-absorptive state—analyses preferably made on serum. Precautions must be taken to avoid any trace of hemolysis. Diazotization must be completed within two hours after collection of the sample.

TEST PROCEDURE

Special Apparatus

Ostwald-Folin pipettes accurately calibrated "to contain" 2 ml.

Graduated test tubes—25 ml in 0.5 ml; the 20 ml graduation must be accurately calibrated.

Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.

Note: Glassware must be chemically clean in order to prevent air bubbles from clinging to the sides of the tubes.

Reagents

1. **Hydrochloric Acid, 1.5% by Volume.** Dilute 15 ml concentrated hydrochloric acid (HCl—Baker & Adamson, A.C.S.) to 1000 ml with distilled water.
2. **Diazo Reagent (Ehrlich).** Immediately prior to use, mix 20 ml sulfanilic acid solution (a) with 0.6 ml 0.5% sodium nitrite (b). Do not keep in a warm place and use within 5 minutes after mixing.
   (a) Sulfanilic acid solution (A); in a 1000 ml mixing cylinder place 1 gm sulfanilic acid ($C_6H_4(NH_2)(SO_3H) \cdot H_2O$ — Mallinckrodt, A.C.S.); add 15 ml concentrated hydrochloric acid (HCl—Baker & Adamson, A.C.S.); dilute to volume with distilled water; shake at intervals until solution is complete and filter. Stability indefinite.
   (b) Sodium nitrite, 0.5% aqueous (B); in a 100 ml volumetric flask place 0.5 gm granular sodium nitrite ($NaNO_2$—Mallinckrodt, A.C.S.); dissolve in distilled water and dilute to volume. Prepare fresh daily.
3. **Methyl Alcohol** (CH_3OH—Merck, A.C.S.).

Technic

Note: For concentrations of bilirubin in excess of 10 mg per 100 ml, dilute 1 ml sample to 20 ml in Step 2 and multiply the values obtained from the calibration table by 2.

1. In a 25 ml graduated test tube place 10 ml distilled water.
2. Add 2 ml sample from an Ostwald-Folin pipette, thoroughly rinsing out the pipette by drawing in and expelling some of the mixture several times, taking care to cause as little foam formation as possible.
3. Dilute to 20 ml with distilled water and mix by inversion.
4. In a cuvette marked "A" (sample blank) place
   5 ml distilled water
   1 ml 1.5% hydrochloric acid (1).
5. In a second cuvette marked "B" (sample) place
   5 ml distilled water
   1 ml freshly prepared diazo reagent (2).
6. In a third cuvette marked "C" (sample blank) place
   5 ml methyl alcohol (3).
   1 ml 1.5% hydrochloric acid (1).
7. In a fourth cuvette marked "D" (sample) place
   5 ml methyl alcohol (3)
   1 ml freshly prepared diazo reagent (2).
8. To each cuvette add 4 ml diluted sample and *note time.*
9. Stopper with clean rubber stoppers and mix by gentle inversion; allow air bubbles to escape—if any remain when transmittancy is to be measured, remove by tapping the cuvettes or by brushing off the walls of the cuvettes with clean applicators.
10. *Exactly* one minute after the addition of the diluted sample, measure the transmittancy of the sample in cuvette "B" against the sample blank in cuvette "A" set at 100% transmission at a wave length of 535 mμ and obtain the concentration of prompt direct-reacting bilirubin from the calibration table—record as result (a).
11. *Exactly* 15 minutes after the addition of the diluted sample, repeat the above transmittancy measurement and obtain the concentration of total direct-reacting bilirubin from the calibration table—record as result (b).

12. *Exactly* 30 minutes after the addition of the diluted sample, measure the transmittancy of the sample in cuvette "D" against the sample blank in cuvette "C" set at 100% transmission at a wave length of 535 mμ and obtain the concentration of total bilirubin from the calibration table—record as result (c). After several minutes repeat the reading to make certain that the maximal value has been attained.

Calculations

Prompt Direct-Reacting Bilirubin—obtained directly from calibration table as result (a).

Total Direct-Reacting Bilirubin—obtained directly from calibration table as result (b).

Delayed Direct-Reacting Bilirubin—subtract the prompt direct-reacting bilirubin (result (a)) from the total direct-reacting bilirubin (result (b)).

Percentage Promptness of the Direct Reaction—divide result (a), the value of the prompt direct-reacting bilirubin at the end of one minute by result (b), the value of the total direct-reacting bilirubin at the end of 15 minutes, and multiply by 100.

Total Bilirubin—obtained directly from calibration table as result (c).

Indirect Bilirubin—subtract the total direct-reacting bilirubin (result (b)) from the total bilirubin (result (c)).

CALIBRATION PROCEDURE

Special Apparatus

Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.

Pipettes calibrated for chloroform, 1 ml.

Reagents

2. **Diazo Reagent (Ehrlich).** Same as for Test Procedure.
3. **Methyl Alcohol.** Same as for Test Procedure.
4. **Phenol-Chloroform Solution (Hubbard-Heilbrun).** In a 250 ml glass-stoppered Erlenmeyer flask place 11 gm crystalline phenol (C_6H_5OH—Baker & Adamson, Acid Carbolic, Reagent, Loose Crystals); add 110 ml anhydrous chloroform ($CHCl_3$—Baker & Adamson, A.C.S.); shake until the crystals are completely dissolved. Prepare just before use.
5. **Bilirubin Standards**

Note: When preparing any standard with chloroform as a solvent, note the temperature and always bring the standard to this temperature before use. In the author's laboratory a temperature of 21°C. is always maintained when working with chloroform solutions.

   (a) Bilirubin stock standard (1 ml contains 0.5 mg bilirubin). Transfer exactly 50 mg bilirubin ($C_{33}H_{36}N_4O_6$—Eastman #2101) to a dry 100 ml volumetric flask thru a small dry funnel, washing off the weighing crystal and funnel with phenol-chloroform solution (4) and dilute to volume with same; shake thoroughly until all the material is completely dissolved. Preserve in a tightly stoppered brown glass bottle. Solution remains stable for at least six months in the refrigerator; a slight deepening of the original tint does not affect the intensity of the color developed upon diazotization.

   (b) Dilute standard (1 ml contains 0.01 mg bilirubin). By means of a pipette calibrated for chloroform, transfer 1 ml stock standard (5a) to a 50 ml volumetric flask; dilute to volume with methyl alcohol (3). Prepare just before use.

   (c) Calibration standards. In a series of four cuvettes place respectively 0, 2, 4 and 6 ml of the dilute standard (5b); add 9, 7, 5 and 3 ml methyl alcohol (3) respectively, bringing the volume in each cuvette to 9 ml. These standards represent concentrations of bilirubin equivalent to 0, 5, 10 and 15 mg per 100 ml sample. Prepare just before use and proceed immediately as follows.

Technic

1. To the cuvettes containing the calibration standards (5c) add 1 ml diazo reagent (2).
2. Stopper with clean rubber stoppers and mix thoroughly.
3. Allow to stand for 30 minutes.
4. Set the zero concentration standard at 100% transmission at a wave length of 535 mμ and record the transmittancy of the 5, 10 and 15 mg per cent standards. After several minutes repeat the readings to make certain that the maximal values have been attained. The transmittancy of the 15 mg standard should be approximately 26%.
5. Plot the observed values on semi-log graph paper—the points fall on a straight line which passes through the origin (Calibration Curve B-10).
6. Prepare a calibration table from the T-C values obtained from the plotted curve.

PROCEDURE FOR USE WITH LINEAR-SCALE PHOTOELECTRIC COLORIMETERS (SHEARD-SANFORD TYPE)

TEST PROCEDURE

Exactly as outlined; set the sample blanks at 100 and use a green filter.

CALIBRATION PROCEDURE

Exactly as outlined—use a green filter; plot the observed readings on semi-log graph paper—the points fall on a straight line passing through the origin.

PROCEDURE FOR USE WITH LOGARITHMIC-SCALE PHOTOELECTRIC COLORIMETERS (KLETT-SUMMERSON TYPE)

TEST PROCEDURE

Exactly as outlined; set the sample blanks at 0 and use a green filter.

CALIBRATION PROCEDURE

Exactly as outlined—use a green filter; plot the observed readings on linear graph paper—the points fall on a straight line passing through the origin.

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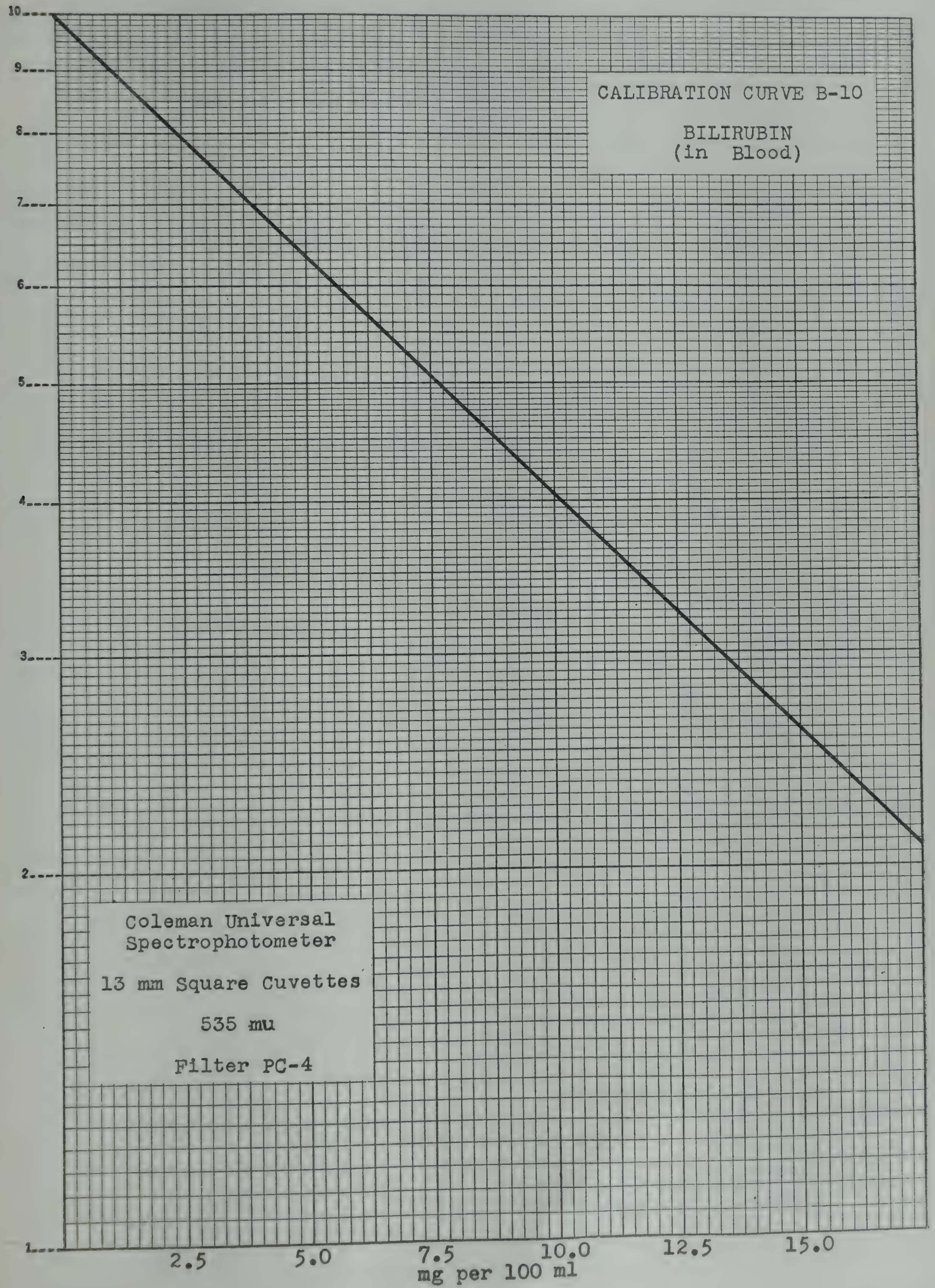
10
9
8
7
6
5
4
3
2
1
CALIBRATION CURVE B-10
BILIRUBIN
(in Blood)
Coleman Universal
Spectrophotometer
13 mm Square Cuvettes
535 mu
Filter PC-4
2.5
5.0
7.5
10.0
12.5
15.0
mg per 100 ml

Meter Reading	0	1	2	3	4	5	6	7	8	9
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BILIRUBIN

A spectrophotometric adaptation based on the methods and modifications of Van den Bergh and Snapper (1913), Van den Bergh and Muller (1916), Davis and Sheard (1934), Malloy and Evelyn (1937), Hubbard and Heilbrun (1941) and Golden and Snavely (1948)

PRINCIPLE

Total bilirubin is determined in alcoholic solution by combination which diazotized sulfanilic acid and subsequent acidification to form a purple derivative of azobilirubin. Since urine, unlike serum, contains interfering chromogenic substances, the optical density of the purple azobilirubin is measured at wave lengths of **575** and **450** mμ, the former being the wave length of maximum absorption of acidified diazotized bilirubin in pure solution and the latter the wave length of maximum absorption of acidified diazotized bilirubin in a mixture. The concentration of bilirubin is calculated from the ratio of the optical densities at the two wave lengths.

DETERMINATION IN URINE

SAMPLE

Aliquot of a 24-hour specimen or a specimen collected over a timed period (the latter is preferable). Keep in stoppered brown bottle in refrigerator until use. Analyses should be made without undue delay. Atabrine interferes with the reaction. This method is unsuitable for urine containing more than 100 mg protein per 100 ml.

TEST PROCEDURE

Special Apparatus

Ostwald-Folin pipettes accurately calibrated "to contain" 1 ml.

Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.

Reagents

1. **Methyl Alcohol** (CH_3OH—Merck, A.C.S.).
2. **Diazo Reagent (Ehrlich).** Immediately prior to use, mix 20 ml sulfanilic acid solution (a) with 0.6 ml 0.5% sodium nitrite (b). Do not keep in a warm place and use within 5 minutes after mixing.
   - (a) Sulfanilic acid solution (A); in a 1000 ml mixing cylinder place 1 gm sulfanilic acid ($C_6H_4(NH_2)(SO_3H) \cdot H_2O$ — Mallinckrodt, A.C.S.); add 15 ml concentrated hydrochloric acid (Reagent #3); dilute to volume with distilled water; shake at intervals until solution is complete and filter. Stability indefinite.
   - (b) Sodium nitrite, 0.5% aqueous (B); in a 100 ml volumetric flask place 0.5 gm granular sodium nitrite ($NaNO_2$—Mallinckrodt, A.C.S.); dissolve in distilled water and dilute to volume. Prepare fresh daily.
3. **Hydrochloric Acid, Concentrated** (HCl—Baker & Adamson, A.C.S.).

Technic

Note: Ordinarily, dilution of the sample to a total volume equivalent to 100 ml per hour is sufficient—for specimens containing high concentrations of bilirubin greater dilutions must be employed.

1. Measure the volume of the 24-hour specimen or that of a sample collected over a timed period.
2. Dilute to a volume that is equivalent to 100 ml per hour—when the volume voided exceeds 100 ml per hour no dilution need be made unless the concentration of bilirubin is excessive.
3. Filter a small portion of the thoroughly mixed diluted urine—if not crystal clear, centrifuge.
4. In a cuvette (sample) place
   8 ml methyl alcohol (1)
   1 ml filtered urine from an Ostwald-Folin pipette, thoroughly rinsing out the pipette by drawing in and expelling some of the mixture several times.
5. In another cuvette (reagent blank) place
   8 ml methyl alcohol (1)
   1 ml distilled water.
6. To each cuvette add 1 ml freshly prepared diazo reagent (2) and mix by lateral shaking.
7. Allow to stand at room temperature for 30 minutes.
8. Add 0.25 ml concentrated hydrochloric acid (3) and mix.
9. Measure the optical density (red scale) of the sample against the reagent blank set at 0 density at a wave length of **575** mμ. Record as result (a).
10. Measure the optical density (red scale) of the sample against the reagent blank set at 0 density at a wave length of **450** mμ. Record as result (b).
11. Calculate the concentration of bilirubin as follows.

Calculation

(1.05 × optical density of sample at 575 mμ (result (a)) − (0.202 × optical density of sample at 450 mμ (result (b)) = Optical density of bilirubin at 575 mμ. Record as result (c)

Refer to the Transmission-Optical Density Chart (see Appendix, Chart X-1) and obtain the transmittancy reading equivalent to the calculated optical density at 575 mμ (result (c)).

Obtain the value of bilirubin equivalent to this transmittancy reading from the calibration table.

Correction for dilution—

$$\frac{\text{Total volume of diluted urine (from Step 2)}}{\text{Original volume (from Step 1)}} \times \text{Value from calibration table} =$$

Mg bilirubin per 100 ml urine

Note: For derivation of factors consult the original article by Golden and Snavely (1948).

CALIBRATION PROCEDURE

Special Apparatus

Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards. Pipettes calibrated for chloroform, 1 ml.

Reagents

1. **Methyl Alcohol.** Same as for Test Procedure.
2. **Diazo Reagent (Ehrlich).** Same as for Test Procedure.
3. **Hydrochloric Acid, Concentrated.** Same as for Test Procedure.
4. **Phenol-Chloroform Solution (Hubbard-Heilbrun).** In a 250 ml glass-stoppered Erlenmeyer flask place 11 gm crystalline phenol (C_6H_5OH—Baker & Adamson, Acid Carbolic, Reagent, Loose Crystals); add 110 ml anhydrous chloroform ($CHCl_3$—Baker & Adamson, A.C.S.); shake until the crystals are completely dissolved. Prepare just before use.
5. **Bilirubin Standards**

Note: When preparing any standard with chloroform as a solvent, note the temperature and always bring the standard to this temperature before use. In the author's laboratory a temperature of 21° C. is always maintained when working with chloroform solutions.

(a) Bilirubin stock standard (1 ml contains 0.5 mg bilirubin). Transfer exactly 50 mg bilirubin ($C_{33}H_{36}N_4O_6$—Eastman #2101) to a dry 100 ml volumetric flask through a small dry funnel, washing off the weighing crystal and funnel with phenol-chloroform solution (4) and dilute to volume with same; shake thoroughly until all the material is completely dissolved. Preserve in a tightly stoppered brown glass bottle. Solution remains stable for at least six months in the refrigerator; a slight deepening of the original tint does not affect the intensity of the color developed upon diazotization.

(b) Dilute standard (1 ml contains 0.01 mg bilirubin). By means of a pipette calibrated for chloroform, transfer 1 ml stock standard (5a) to a 50 ml volumetric flask; dilute to volume with methyl alcohol (1). Prepare just before use.

(c) Calibration standards. In a series of seven cuvettes place respectively 0, 0.5, 1, 2, 3, 4 and 5 ml of the dilute standard (5b); add 8, 7.5, 7, 6, 5, 4 and 3 ml methyl alcohol (1) respectively, bringing the volume in each cuvette to 8 ml. These standards represent concentrations of bilirubin equivalent to 0, 0.5, 1, 2, 3, 4 and 5 mg per 100 ml sample. Prepare just before use and proceed immediately as follows.

Technic

1. To the cuvettes containing the calibration standards (5c) add 1 ml distilled water and mix by lateral shaking.
2. Add 1 ml diazo reagent (2) and mix thoroughly by lateral shaking.
3. Allow to stand for 30 minutes.
4. Set the zero concentration standard at 100% transmission at a wave length of 575 mμ and record the transmittancy of the 0.5, 1, 2, 3, 4 and 5 mg per cent standards. After several minutes repeat the readings to make certain that the maximal values have been attained. The transmittancy of the 5 mg standard should be approximately 25%.
5. Plot the observed values on semi-log graph paper—the points fall on a straight line which passes through the origin (Calibration Curve B-11).

6. Prepare a calibration table from the T-C values obtained from the plotted curve.

PROCEDURE FOR USE WITH PHOTOELECTRIC COLORIMETERS (DOUBLE-SCALE TYPE)

TEST PROCEDURE

Exactly as outlined; set the reagent blank at 0 density and measure the optical density with an orange filter and then with a green filter. When calculating the results, the equation given previously cannot be used—the analyst must determine the numerical factors for his own instrument (see original article).

CALIBRATION PROCEDURE

Exactly as outlined—use an orange filter; plot the observed readings on the appropriate type of graph paper—the points fall on a straight line passing through the origin.

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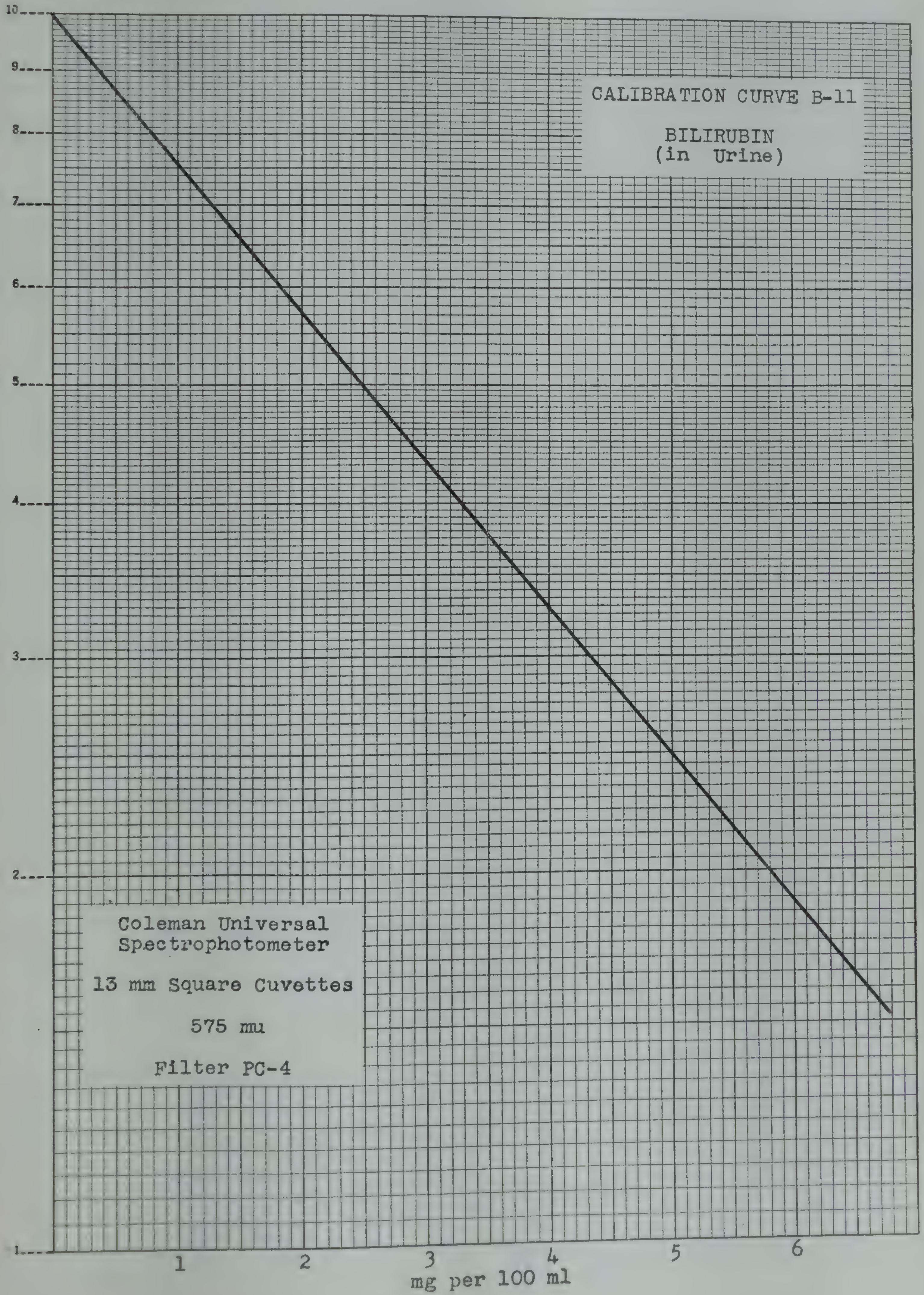
CALIBRATION CURVE B-11
BILIRUBIN
(in Urine)
Coleman Universal Spectrophotometer
13 mm Square Cuvettes
575 mu
Filter PC-4
10
9
8
7
6
5
4
3
2
1
1
2
3
4
5
6
mg per 100 ml

Meter Reading	0	1	2	3	4	5	6	7	8	9
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10										
20										
30										
40										
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70										
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BISMUTH

A spectrophotometric adaptation based on the methods and modifications of Lehman, Richardson and Hanzlik (1935) and Hanzlik, Lehman, Richardson and Van Winkle (1937)

PRINCIPLE

Organic matter in urine is destroyed by permanganate oxidation, the color due to excess permanganate being discharged by oxalic acid. Bismuth is determined by the yellow color developed upon treatment with potassium iodide, interference from iron being eliminated by the use of sodium citrate and sodium sulfite.

DETERMINATION IN URINE

SAMPLE

Aliquot of well-mixed 24-hour excretion since individual specimens may be bismuth-free.

TEST PROCEDURE

Special Apparatus

Ignition tubes—Pyrex, 25 × 200 mm; accurately calibrated at the 25 ml mark.
Micro burner.
Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.

Reagents

1. **Potassium Permanganate, 6.6% Aqueous.** Dissolve 6.6 gm potassium permanganate ($KMnO_4$—Baker & Adamson, A.C.S.) in 100 ml distilled water with the aid of heat; allow to cool to room temperature; adjust volume to 100 ml and filter.
2. **Sulfuric Acid, Concentrated** (H_2SO_4—Grasselli, A.C.S.).
3. **Oxalic Acid, 10% Aqueous.** Dissolve 10 gm crystalline oxalic acid ($H_2C_2O_4 \cdot 2H_2O$—Mallinckrodt, A.C.S.) in 100 ml distilled water with the aid of heat; allow to cool to room temperature; adjust volume to 100 ml and filter.
4. **Sodium Sulfite, 10% Aqueous.** Dissolve 10 gm crystalline sodium sulfite ($Na_2SO_3 \cdot 7H_2O$—J. T. Baker, CP Analyzed) in distilled water and dilute to 100 ml.
5. **Sodium Citrate, 15% Aqueous.** Dissolve 15 gm crystalline sodium citrate ($Na_3C_6H_5O_7 \cdot 2H_2O$—Baker & Adamson, Reagent) in distilled water and dilute to 100 ml.
6. **Potassium Iodide, 20% Aqueous.** Dissolve 10 gm potassium iodide (KI—J. T. Baker, CP Analyzed, Low in Iodates, Special) in distilled water and dilute to 50 ml. This solution must show no trace of free iodine when tested with starch indicator solution and should be prepared in quantities sufficient for immediate use only.

Technic

Note: For concentrations of bismuth less than 0.15 mg per 100 ml, take a 50 ml aliquot and evaporate to a volume of 10 ml or less before proceeding with Step 1 and divide the values obtained from the calibration table by 2.5.

1. In a Pyrex ignition tube (sample) place 20 ml thoroughly mixed unfiltered urine and evaporate on a steam bath to slightly less than one-half volume.
2. Add 6 ml 6.6% potassium permanganate (1), mix and add
    2 ml concentrated sulfuric acid (2), slowly and with constant gentle agitation of the tube, allowing the acid to fall directly into the fluid.
3. Heat to boiling with a micro burner and boil gently for at least 2 minutes—control foaming by constantly playing the flame up and down the side of the tube.
4. While still hot, slowly add 5 ml 10% oxalic acid (3) and mix—if not completely decolorized, heat gently for 1 minute, and if still not completely decolorized, repeat digestion with another 6 ml portion of permanganate, again decolorize with 5 ml oxalic acid and boil until the volume is less than 18 ml.
5. In another Pyrex ignition tube (reagent blank) place
    5 ml distilled water
    6 ml 6.6% potassium permanganate (1) and mix
    2 ml concentrated sulfuric acid (2), added as above.
6. Heat to boiling with a micro burner and decolorize with 5 ml 10% oxalic acid (3).
7. Allow both tubes to cool to room temperature.
8. To each tube add
    0.5 ml 10% sodium sulfite (4) and mix
    1 ml 15% sodium citrate (5) and mix
    0.5 ml 20% freshly prepared potassium iodide (6) and mix.
9. Dilute to 25 ml with distilled water and mix thoroughly.
10. Allow to stand at room temperature for 5 minutes.
11. Measure the transmittancy of the sample against the reagent blank set at 100% transmission at a wave length of 400 mμ and obtain the concentration of bismuth from the calibration table.

CALIBRATION PROCEDURE

Special Apparatus

Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.

Graduated test tubes—25 ml in 0.5 ml; the 25 ml graduation must be accurately calibrated.

Reagents

2. **Sulfuric Acid, Concentrated.** Same as for Test Procedure.
4. **Sodium Sulfite, 10% Aqueous.** Same as for Test Procedure.
5. **Sodium Citrate, 15% Aqueous.** Same as for Test Procedure.
6. **Potassium Iodide, 20% Aqueous.** Same as for Test Procedure.
7. **Nitric Acid, 50% by Volume.** Dilute 50 ml concentrated nitric acid (HNO_3—Grasselli, A.C.S.) with an equal volume of distilled water and mix.
8. **Bismuth Standards**
   (a) Bismuth stock standard (1 ml contains 1 mg bismuth). Dry bismuth trioxide (Bi_2O_3—Reagent Chemicals, Special) at 110° C. to constant weight; transfer 115 mg to a small beaker and dissolve in 5 ml 50% nitric acid (7) with the aid of heat; allow to cool and transfer quantitatively to a 100 ml volumetric flask; add 5 ml 50% nitric acid and dilute to volume with distilled water.
   (b) Dilute standard (1 ml contains 0.08 mg bismuth). In a 50 ml volumetric flask place 4 ml stock standard (8a); add approximately 40 ml distilled water and a few drops of 50% nitric acid (7); dilute to volume with distilled water.
   (c) Calibration standards. In a series of six accurately calibrated 10 ml volumetric flasks place respectively 0, 2, 4, 6, 8 and 10 ml of the dilute standard (8b); to each flask except the last in the series add 1 drop of 50% nitric acid (7) and dilute to volume with distilled water. These standards represent concentrations of bismuth equivalent to 0, 0.4, 0.8, 1.2, 1.6 and 2.0 mg per 100 ml sample. Prepare just before use.

Technic

1. Transfer 5 ml of each calibration standard (8c) to a correspondingly marked 25 ml graduated test tube.
2. To each tube add 5 ml distilled water and mix.
3. Add 2 ml concentrated sulfuric acid (2) and mix.
4. Allow to cool to room temperature.
5. Proceed exactly as outlined in the Test Procedure, Steps 8–10 inclusive.
6. Set the zero concentration standard at 100% transmission at a wave length of 400 mμ and record the transmittancy of the 0.4, 0.8, 1.2, 1.6 and 2.0 mg per cent standards. The transmittancy of the 2.0 mg standard should be approximately 26%.
7. Plot the observed values on semi-log graph paper—the points fall on a very slight curve which passes thru the origin (Calibration Curve B-15).
8. Prepare a calibration table from the T-C values obtained from the plotted curve.

PROCEDURE FOR USE WITH LINEAR-SCALE PHOTOELECTRIC COLORIMETERS (SHEARD-SANFORD TYPE)

TEST PROCEDURE

Exactly as outlined; set the reagent blank at 100 and use a blue filter.

CALIBRATION PROCEDURE

Exactly as outlined—use a blue filter; plot the observed readings on semi-log graph paper—the points fall on a very slight curve passing through the origin.

PROCEDURE FOR USE WITH LOGARITHMIC-SCALE PHOTOELECTRIC COLORIMETERS (KLETT-SUMMERSON TYPE)

TEST PROCEDURE

Exactly as outlined; set the reagent blank at 0 and use a blue filter.

CALIBRATION PROCEDURE

Exactly as outlined—use a blue filter; plot the observed readings on linear graph paper—the points fall on a very slight curve passing through the origin.

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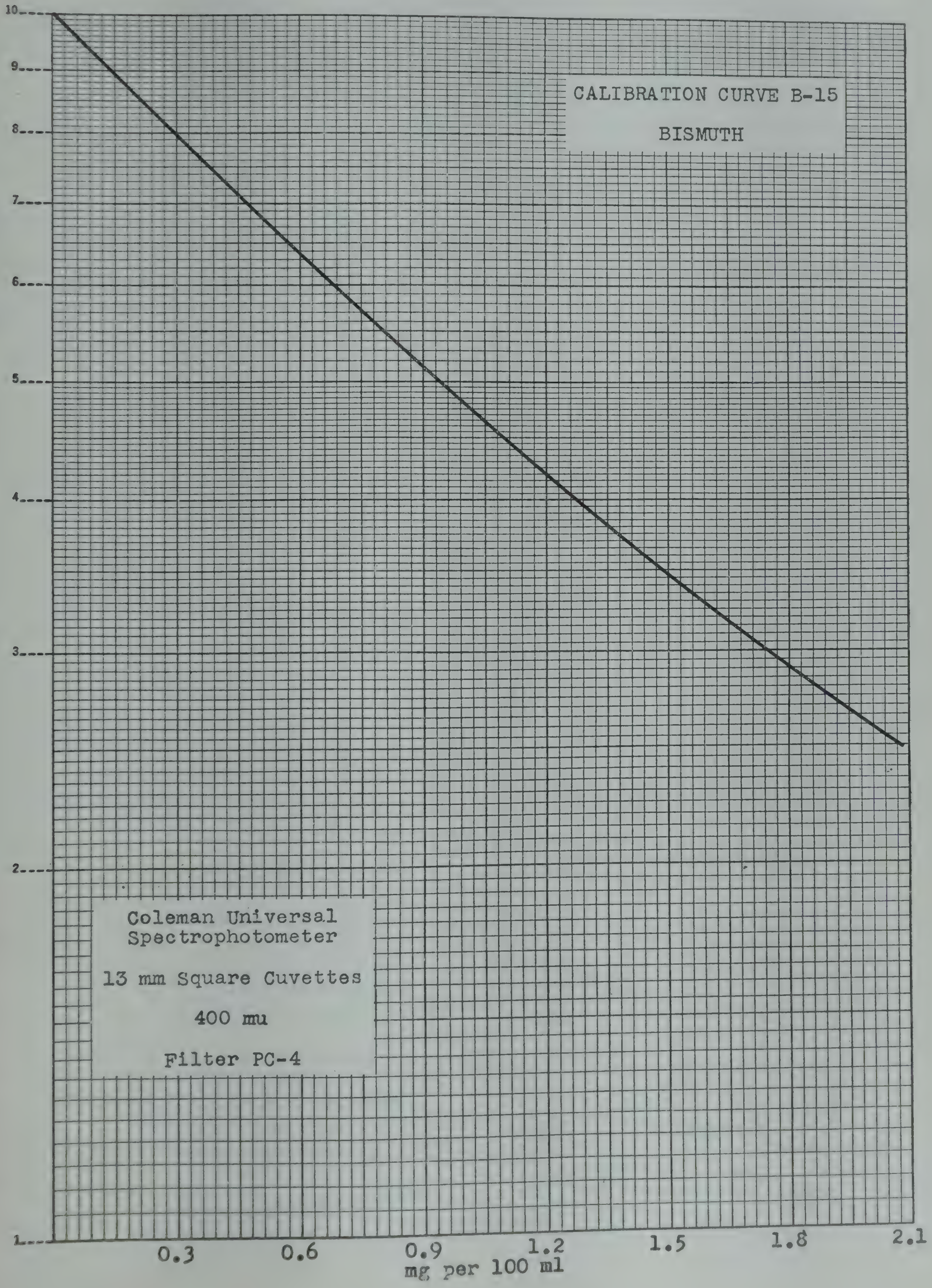
CALIBRATION CURVE B-15
BISMUTH
Coleman Universal Spectrophotometer
13 mm Square Cuvettes
400 mu
Filter PC-4
10
9
8
7
6
5
4
3
2
1
0.3
0.6
0.9
1.2
1.5
1.8
2.1
mg per 100 ml

Meter Reading	0	1	2	3	4	5	6	7	8	9
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BROMIDE

A spectrophotometric adaptation based on the methods and modifications of Walter (1925), Wuth (1927), Taylor (1927), Malamud, Fuchs and Malamud (1928), Diethelm (1930), Katzenelbogen and Goldsmith (1931), Tod (1933), Katzenelbogen and Czarski (1934), Gundry (1939) and Flinn (1941)

PRINCIPLE

Bromide is determined by the brownish-yellow color produced upon treatment of the protein-free trichloracetic acid filtrate with gold chloride. The protein precipitant and the standards contain a definite amount of sodium chloride, and the standards, in addition, contain a trace of iodide, to compensate for the color produced by normal amounts of these substances in the blood.

DETERMINATION IN SERUM

SAMPLE

Analyses should be made on serum only since anticoagulants cause interference with the color development. This method is not suitable for the determination of the normal bromide content of the blood. It should be used only to determine the bromide level during or after the administration of bromides, with due regard to the fact that substances other than bromides are responsible for a part of the final color. It is included here because of its widespread clinical use.

TEST PROCEDURE

Special Apparatus

Ostwald-Folin pipettes accurately calibrated "to deliver" 2 ml.

Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.

Reagents

1. **Trichloracetic Acid-Chloride Solution "A".** Dilute 10 ml concentrated trichloracetic acid (a) to 100 ml with 0.6% sodium chloride (b).
    - (a) Trichloracetic acid, concentrated; dissolve 100 gm crystalline trichloracetic acid (CCl_3-$COOH$—J. T. Baker, CP Analyzed) in distilled water and dilute to 100 ml. Preserve in Pyrex glass-stoppered bottle. Stability indefinite.
    - (b) Sodium chloride, 0.6% aqueous; dissolve 6 gm sodium chloride (NaCl—Merck, Special for Biological Work) in distilled water and dilute to 1000 ml.
2. **Gold Chloride, 0.5% Aqueous.** Since gold chloride is extremely hygroscopic, it is best to purchase the material in 15 grain ampoules ($AuCl_3 \cdot HCl \cdot 3H_2O$—Merck, Reagent). To prepare the solution, soak the label off the ampoule, break in half with a file and place both parts in a beaker containing exactly 195 ml distilled water; stir until dissolved and filter.

Technic

Note: For concentrations of bromide in excess of 200 mg per 100 ml, dilute 1 volume of sample with 1 volume of 0.6% sodium chloride (1b) before proceeding with Step 1 and multiply the values obtained from the calibration table by 2.

1. In a 15 ml centrifuge tube place
    8 ml trichloracetic acid-chloride solution "A" (1)
    2 ml serum from an Ostwald-Folin pipette, added drop by drop, with constant agitation of the tube.
2. Shake vigorously and allow to stand for 30 minutes.
3. Centrifuge at 1500 rpm for 5 minutes (or filter through a dry 7 cm Whatman #44 paper into a dry tube).
4. In a cuvette (sample) place
    5 ml clear supernatant centrifugate (or filtrate)
    1 ml 0.5% gold chloride (2) and mix
    4 ml distilled water and mix.
5. In another cuvette (reagent blank) place
    4 ml trichloracetic acid-chloride solution "A" (1)
    5 ml distilled water
    1 ml 0.5% gold chloride (2) and mix.
6. Within 10 minutes measure the transmittancy of the sample against the reagent blank set at 100% transmission at a wave length of 440 mμ and obtain the concentration of bromide expressed as sodium bromide from the calibration table.

CALIBRATION PROCEDURE

Special Apparatus

Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.

Reagents

1. **Trichloracetic Acid-Chloride Solution "A".** Same as for Test Procedure.
2. **Gold Chloride, 0.5% Aqueous.** Same as for Test Procedure.
3. **Potassium Iodide, 0.1% Aqueous.** Dissolve 10 mg potassium iodide (KI—J. T. Baker, Low in Iodates, Special) in distilled water and dilute to 10 ml. Prepare just before use.

4. **Bromide Standards**
   (a) Bromide stock standard, Tod (1 ml contains 10 mg sodium bromide). In a 100 ml volumetric flask place 1 gm granular sodium bromide (NaBr—Baker & Adamson, Reagent) and dissolve in approximately 50 ml distilled water; add 0.15 ml 0.1% potassium iodide (3) and dilute to volume with distilled water.
   (b) Dilute standard (1 ml contains 0.3 mg sodium bromide). In a 100 ml volumetric flask place 3 ml stock standard (4a) and dilute to volume with distilled water. Prepare just before use.
   (c) Calibration standards. In a series of six cuvettes place respectively 0, 1, 2, 3, 4 and 5 ml of the dilute standard (4b); add 5, 4, 3, 2, 1 and 0 ml distilled water respectively, bringing the volume in each cuvette to 5 ml. These standards represent concentrations of sodium bromide equivalent to 0, 30, 60, 90, 120 and 150 mg per 100 ml sample. Prepare just before use and proceed immediately as follows.

Technic

1. To each calibration standard (4c) add
   4 ml trichloracetic acid-chloride solution "A" (1) and mix
   1 ml 0.5% gold chloride (2) and mix thoroughly.
2. Within 10 minutes set the zero concentration standard at 100% transmission at a wave length of 440 mμ and record the transmittancy of the 30, 60, 90, 120 and 150 mg per cent standards. The transmittancy of the 150 mg standard should be approximately 39%.
3. Plot the observed values on semi-log graph paper —the points fall on a straight line which passes through the origin (Calibration Curve B-20).
4. Prepare a calibration table from the T-C values obtained from the plotted curve.

CONVERSION FACTORS

To convert sodium bromide in mg per 100 ml to bromide in mg per 100 ml multiply by 0.776.

To convert sodium bromide in mg per 100 ml to potassium bromide in mg per 100 ml multiply by 1.15.

Method B-20b

DETERMINATION IN URINE

SAMPLE

Aliquot of a 24-hour or casual specimen—elimination of bromide is rapid and constant.

TEST PROCEDURE

Special Apparatus

As listed under the Test Procedure for blood.

Reagents

1. **Trichloracetic Acid-Chloride Solution "A".** Same as for blood.
2. **Gold Chloride, 0.5% Aqueous.** Same as for blood.

Technic

Note: See remarks under blood.

1. Filter a small portion of thoroughly mixed urine.
2. In a 15 ml centrifuge tube place
   8 ml trichloracetic acid-chloride solution "A" (1)
   2 ml filtered urine from an Ostwald-Folin pipette, added drop by drop, with constant agitation of the tube.
3. Proceed exactly as outlined in the Test Procedure for blood, Steps 3–6 inclusive.

Method B-20c

DETERMINATION IN CEREBROSPINAL FLUID

SAMPLE

The admixture of whole or hemolyzed blood or the presence of partially decomposed blood vitiates the results.

TEST PROCEDURE

Special Apparatus

Ostwald-Folin pipettes accurately calibrated "to deliver" 4 ml.

Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.

Reagents

2. **Gold Chloride, 0.5% Aqueous.** Same as for blood.
5. **Trichloracetic Acid-Chloride Solution "B".** Dilute 13.3 ml concentrated trichloracetic acid (1a) to 100 ml with 0.75% sodium chloride (a).
   (a) Sodium chloride, 0.75% aqueous; dissolve 7.5 gm sodium chloride (NaCl—Merck, Special for Biological Work) in distilled water and dilute to 1000 ml.

Technic

1. In a 15 ml centrifuge tube place
    - 6 ml trichloracetic acid-chloride solution "B" (5)
    - 4 ml cerebrospinal fluid from an Ostwald-Folin pipette, added drop by drop, with constant agitation of the tube.
2. Proceed exactly as outlined in the Test Procedure for blood, Steps 2–5 inclusive.
3. Within 10 minutes measure the transmittancy of the sample against the reagent blank set at 100% transmission at a wave length of **440** mμ and divide the value from the calibration table for blood by 2 to obtain the concentration of bromide expressed as sodium bromide in mg per 100 ml cerebrospinal fluid.

PROCEDURE FOR USE WITH LINEAR-SCALE PHOTOELECTRIC COLORIMETERS (SHEARD-SANFORD TYPE)

TEST PROCEDURE

Exactly as outlined; set the reagent blank at 100 and use a blue filter.

CALIBRATION PROCEDURE

Exactly as outlined—use a blue filter; plot the observed readings on semi-log graph paper—the points fall on a straight line passing through the origin.

PROCEDURE FOR USE WITH LOGARITHMIC-SCALE PHOTOELECTRIC COLORIMETERS (KLETT-SUMMERSON TYPE)

TEST PROCEDURE

Exactly as outlined; set the reagent blank at 0 and use a blue filter.

CALIBRATION PROCEDURE

Exactly as outlined—use a blue filter; plot the observed readings on linear graph paper—the points fall on a straight line passing through the origin.

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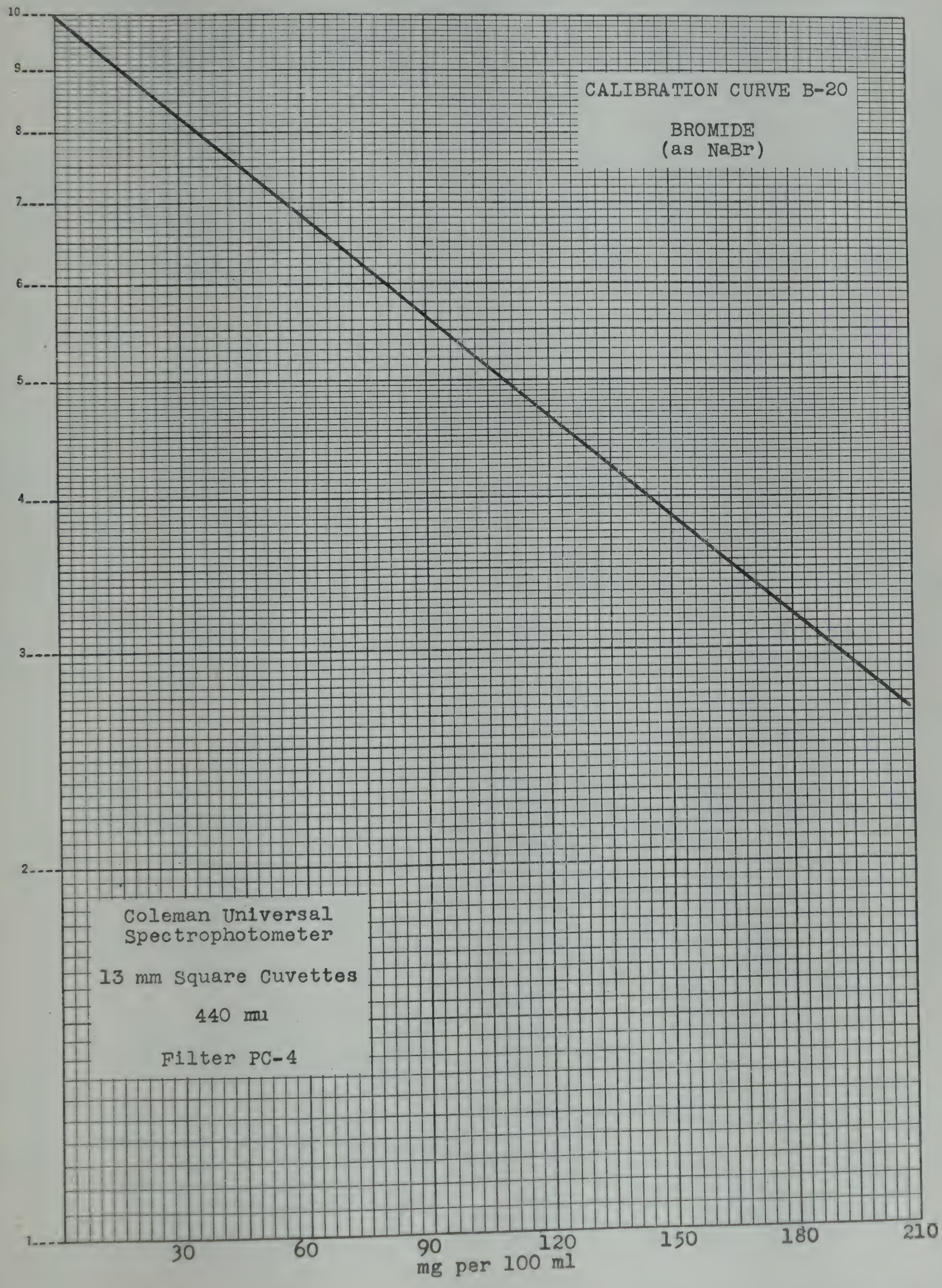
NOTES

NOTES


CALIBRATION CURVE B-20
BROMIDE
(as NaBr)
Coleman Universal Spectrophotometer
13 mm Square Cuvettes
440 mμ
Filter PC-4
10
9
8
7
6
5
4
3
2
1
30
60
90
120
150
180
210
mg per 100 ml

Meter Reading	0	1	2	3	4	5	6	7	8	9
0										
10										
20										
30										
40										
50										
60										
70										
80										
90										

BROMSULPHALEIN

A spectrophotometric adaptation based on the methods and modifications of Rosenthal and White (1925), Greene, Snell and Walters (1925), Pratt, Vanlandingham, Talley, Nelson and Johnson (1932) and Dragstedt and Mills (1936)

PRINCIPLE

Bromsulphalein is colorless in acid solution and reddish-violet in alkaline solution. This color is utilized for its determination by comparison with an arbitrary standard, the latter differing in concentration according to the amount of dye injected in the particular technic employed.

DETERMINATION IN PLASMA OR SERUM

SAMPLE

When carrying out the bromsulphalein hepatic function test, three or more samples are necessary—one from blood withdrawn during post-absorptive state prior to the administration of bromsulphalein and two or more, depending upon the technic followed, from blood secured at definite intervals after the administration of the dye. With the original Rosenthal technic 2 mg bromsulphalein per kilogram of body weight are injected; with the modified Greene technic 5 mg of dye per kilogram of body weight are injected. For plasma, heparin is preferable to potassium oxalate as an anticoagulant. Adequate precautions must be taken to avoid any trace of hemolysis. Sera containing excessive amounts of fats or biliary pigments yield inaccurate results.

TEST PROCEDURE

Special Apparatus

Ostwald-Folin pipettes accurately calibrated "to contain" 1 ml.
Graduated test tubes—10 ml in 0.1 ml; the 10 ml graduation must be accurately calibrated.
Capillary droppers delivering 40 drops per ml.
Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.

Reagents

1. **Sodium Chloride, 0.9% Aqueous.** Dissolve 9 gm sodium chloride (NaCl—Merck, Special for Biological Work) in distilled water and dilute to 1000 ml.
2. **Sodium Hydroxide, Volumetric 2.5 N.** Prepare by diluting slightly more than the required amount of concentrated carbonate-free sodium hydroxide solution (see Appendix); dilute 20 ml of this approximately 2.5 N solution to 100 ml; titrate against standard 0.5 N acid using phenolphthalein as indicator; adjust accordingly and re-titrate—20 ml of 0.5 N acid require 20 ml of a 1:5 dilution of 2.5 N alkali. Preserve in ceresin-lined bottle.
3. **Hydrochloric Acid, 5% by Volume.** Dilute 5 ml concentrated hydrochloric acid (HCl—Baker & Adamson, A.C.S.) to 100 ml with distilled water.

Technic

Note: For concentrations of bromsulphalein in excess of 75%, use 0.5 ml sample in Steps 1–3 inclusive and multiply the values obtained from the calibration table by 2.

1. In a 10 ml graduated test tube (sample blank) place
   - 8 ml 0.9% sodium chloride (1)
   - 1 ml plasma or serum from blood withdrawn prior to the administration of bromsulphalein, from an Ostwald-Folin pipette, thoroughly rinsing out the pipette by drawing in and expelling some of the mixture several times
   - 1 drop 5% hydrochloric acid (3), delivered from a capillary-tipped dropper.
2. In another 10 ml graduated test tube (sample I) place
   - 8 ml 0.9% sodium chloride (1)
   - 1 ml plasma or serum from the first blood specimen withdrawn after the administration of the dye, from an Ostwald-Folin pipette in the same manner as before
   - 1 drop 2.5 N sodium hydroxide (2), delivered from a capillary-tipped dropper.
3. In similar 10 ml graduated test tubes place 8 ml 0.9% sodium chloride (1), 1 ml plasma or serum from each additional blood specimen and 1 drop 2.5 N sodium hydroxide (2).
4. To each tube add sufficient 0.9% sodium chloride (1) to bring the volume to 10 ml and mix.
5. Measure the transmittancy of the samples against the sample blank set at 100% transmission at a wave length of 575 mμ and obtain the concentrations of bromsulphalein from the calibration table.

CALIBRATION PROCEDURE

Special Apparatus

Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.

Reagents

4. **Sodium Hydroxide, Volumetric 0.005 N.** Dilute 0.2 ml 2.5 N sodium hydroxide (2) to 100 ml with distilled water. Preserve in ceresin-lined bottle.

5. **Bromsulphalein Standards**
   - (a) Bromsulphalein stock standard (1 ml contains 1 mg bromsulphalein). In a 100 ml volumetric flask place 2 ml bromsulphalein ($C_8Br_4O_2(C_6H_3OHSO_3Na)_2$—Hynson, Westcott and Dunning; furnished in ampoules of 3 ml, each ml containing 50 mg of the dye) and dilute to volume with 0.005 N sodium hydroxide (4). Preserve in ceresin-lined bottle.
   - (b) Dilute standard (1 ml contains 0.01 mg bromsulphalein). In a 100 ml volumetric flask place 1 ml stock standard (5a) and dilute to volume with 0.005 N sodium hydroxide (4). Prepare just before use.
   - (c) Calibration standards. In a series of five cuvettes place respectively 0, 1, 3, 5 and 7 ml of the dilute standard (5b); add 10, 9, 7, 5 and 3 ml 0.005 N sodium hydroxide (4) respectively, bringing the volume in each cuvette to 10 ml. These standards represent concentrations of bromsulphalein equivalent to 0, 10, 30, 50 and 70% when a 100 mg per liter solution is designated as "100%." Prepare just before use and proceed immediately as follows.

Technic

1. Set the zero concentration standard at 100% transmission at a wave length of **575** mμ and record the transmittancy of the 10, 30, 50 and 70% standards (5c). The transmittancy of the 70% standard should be approximately 27%.
2. Plot the observed values on semi-log graph paper—the points fall on a very slight curve which passes through the origin (Calibration Curve B-25).
3. Prepare a calibration table from the T-C values obtained from the plotted curve.

Note: The calibration procedure outlined is based on the administration of 5 mg dye per kilogram of body weight with a 100 mg per liter solution designated as "100%." When the Rosenthal technic is employed (2 mg per kilogram), multiply the values obtained from the calibration table by 2.5.

PROCEDURE FOR USE WITH LINEAR-SCALE PHOTOELECTRIC COLORIMETERS (SHEARD-SANFORD-TYPE)

TEST PROCEDURE

Exactly as outlined; set the sample blank at 100 and use a green filter.

CALIBRATION PROCEDURE

Exactly as outlined—use a green filter; plot the observed readings on semi-log graph paper—the points fall on a slight curve passing through the origin.

PROCEDURE FOR USE WITH LOGARITHMIC-SCALE PHOTOELECTRIC COLORIMETERS (KLETT-SUMMERSON TYPE)

TEST PROCEDURE

Exactly as outlined; set the sample blank at 0 and use a green filter.

CALIBRATION PROCEDURE

Exactly as outlined—use a green filter; plot the observed readings on linear graph paper—the points fall on a straight line passing through the origin.

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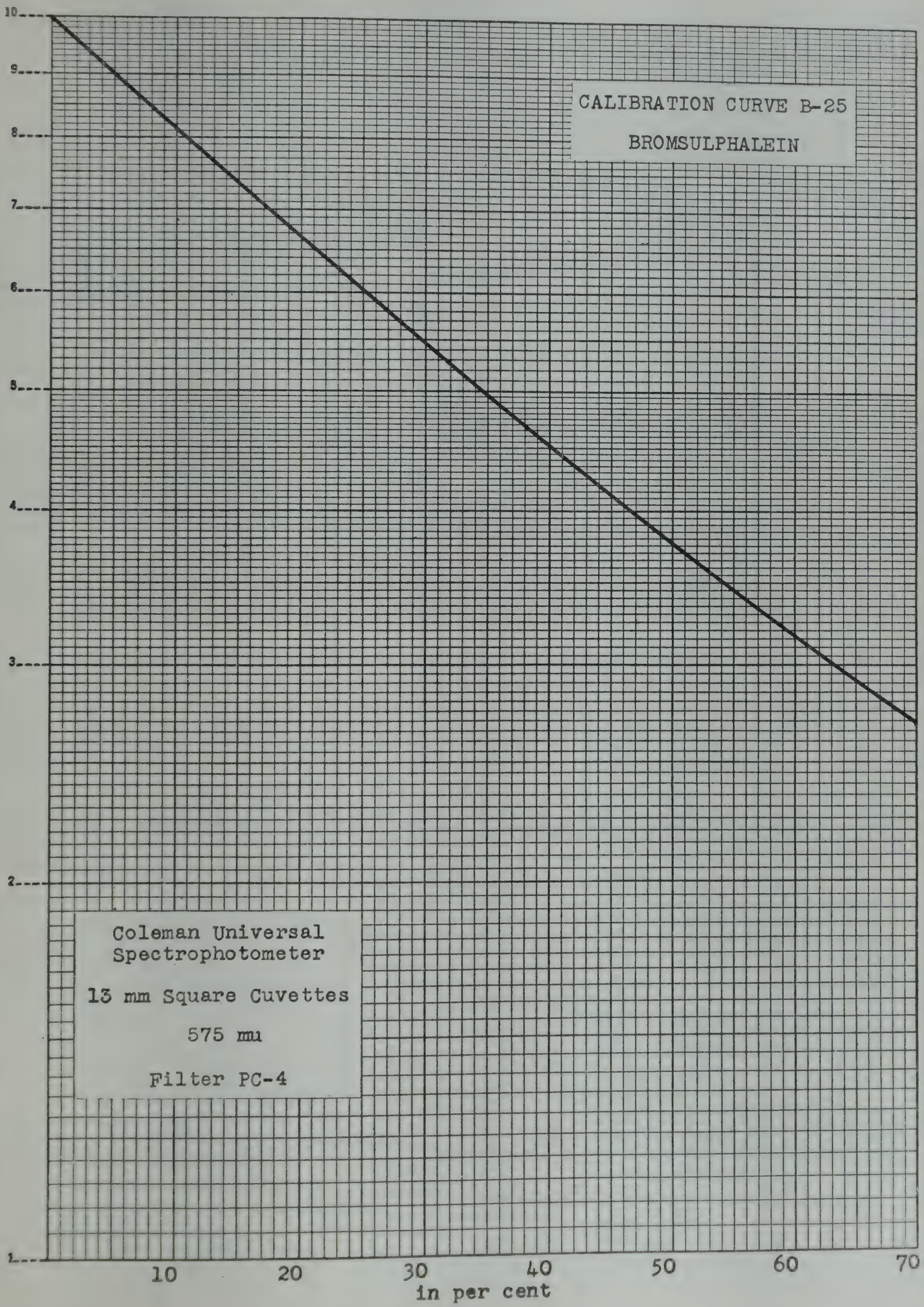
CALIBRATION CURVE B-25
BROMSULPHALEIN
Coleman Universal
Spectrophotometer
13 mm Square Cuvettes
575 mu
Filter PC-4
10
9
8
7
6
5
4
3
2
1
10
20
30
40
50
60
70
in per cent

Meter Reading	0	1	2	3	4	5	6	7	8	9
0										
10										
20										
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60										
70										
80										
90										

CALCIUM

A spectrophotometric adaptation based on the methods and modifications of Shohl and Pedley (1922), Clark and Collip (1925), Fiske and SubbaRow (1925), Roe and Kahn (1926; 1929), Wang (1935), Roe and Whitmore (1938) and Hoffman (1941)

PRINCIPLE

Calcium is precipitated as the phosphate, which is dissolved in trichloracetic acid and the phosphorus determined as outlined in the method for inorganic phosphorus (Method P-36), the same analytical principles being involved.

DETERMINATION IN PLASMA OR SERUM

SAMPLE

Serum or plasma from blood withdrawn during post-absorptive state. Serum must be removed from clot as quickly as possible by centrifugation. There must be no trace of hemolysis. The serum may be preserved for several hours in the refrigerator or for a longer period by the addition of a drop of toluene. The analysis should not be unduly delayed. For calcium determinations in plasma heparin should be used as an anticoagulant. Analyses preferably made on serum.

TEST PROCEDURE

Special Apparatus

Centrifuge tubes—15 ml, glass-stoppered.
Ostwald-Folin pipettes accurately calibrated "to deliver" 1 ml.
Filter paper, double acid-washed, calcium and phosphorus-free, Whatman #44, 7 cm (required only if filtrates instead of centrifugates are employed).
Calcium tubes—Hoffman-Fister, accurately calibrated at the 8 ml graduation.
Stirring rods, glass, platinum-tipped.
Wash bottles, all-glass, Pyrex, 125 ml.
Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.

Note: Glassware must be calcium and phosphorus-free. It should be thoroughly cleaned with dichromate-sulfuric acid solution (see Appendix), rinsed with distilled water and dried before use.

Reagents

1. **Trichloracetic Acid, 5% Aqueous.** Dilute 5 ml concentrated trichloracetic acid (a) to 100 ml with distilled water. Prepare fresh weekly.
   (a) Trichloracetic acid, concentrated; dissolve 100 gm crystalline trichloracetic acid (CCl_3-$COOH$—J. T. Baker, CP Analyzed) in distilled water and dilute to 100 ml. Preserve in Pyrex glass-stoppered bottle. Stability indefinite.
2. **Sodium Hydroxide, Volumetric 6.25 N.** Prepare by diluting slightly more than the required amount of concentrated carbonate-free sodium hydroxide solution (see Appendix); dilute 8 ml of this approximately 6.25 N solution to 100 ml; titrate against standard 0.5 N acid using phenolphthalein as indicator; adjust accordingly and re-titrate—20 ml of 0.5 N acid require 20 ml of a 1:12.5 dilution of 6.25 N alkali. Preserve in ceresin-lined bottle. Note: When prepared in this manner the hydroxide is calcium-free.
3. **Sodium Phosphate, Tribasic, 1% Aqueous.** Dissolve 1 gm tribasic sodium phosphate ($Na_3PO_4 \cdot 12H_2O$—Merck, Reagent) in distilled water and dilute to 100 ml. Preserve in ceresin-lined bottle.
4. **Alkaline Alcohol Wash Solution (Roe-Kahn).** In a 100 ml mixing cylinder place 58 ml absolute methyl alcohol (CH_3OH—Merck, A.C.S.), 10 ml iso-amyl alcohol ($C_5H_{11}OH$—Baker & Adamson, A.C.S.) and dilute to volume with distilled water; add 2 drops 1% phenolphthalein (a) and 3 drops 5% sodium hydroxide (b)—a distinct pink color appears. Preserve and use in an all-glass Pyrex wash bottle.
   (a) Phenolphthalein, 1% alcoholic; dissolve 1 gm phenolphthalein ($C_{20}H_{14}O_4$—Baker & Adamson, A.C.S.) in 100 ml absolute methyl alcohol (CH_3OH—Merck, A.C.S.).
   (b) Sodium hydroxide, 5 % aqueous; dilute 20 ml 6.25 N sodium hydroxide (2) to 100 ml with distilled water. Preserve in ceresin-lined bottle.
5. **Molybdate Reagent II (Fiske-SubbaRow).** In a 100 ml volumetric flask place 30 ml 10 N sulfuric acid (a); add 20 ml 12.5% ammonium molybdate (b) and dilute to volume with distilled water. Preserve in Pyrex glass-stoppered bottle.
   (a) Sulfuric acid, volumetric 10 N; slowly add 300 ml concentrated sulfuric acid (H_2SO_4—Grasselli, A.C.S.) to 750 ml distilled water; mix well and allow to cool; dilute 10 ml of this approximately 10 N solution to 100 ml; titrate against standard 1 N alkali using phenolphthalein as indicator; adjust accordingly and re-titrate—20 ml of a 1:10 dilution of 10 N acid require 20 ml of 1 N alkali. Preserve in Pyrex glass-stoppered bottle.
   (b) Ammonium molybdate, 12.5% aqueous; dissolve 2.5 gm ammonium molybdate ($(NH_4)_6Mo_7O_{24} \cdot 4H_2O$—Mallinckrodt, AR)

in distilled water and dilute to 20 ml. If larger quantities are prepared, preserve in ceresin-lined bottle. Discard when precipitate forms.

6. **Aminonaphthol Sulfonic Acid Reagent (Fiske-SubbaRow).** In a 100 ml mixing cylinder place 97.5 ml 15% sodium bisulfite (a) and 2.5 ml 20% sodium sulfite (b); add 250 mg recrystallized 1-amino-2-naphthol-4-sulfonic acid (NH_2-$C_{10}H_5(OH)SO_3H$—Reagent Chemicals, Special) and shake until dissolved. With a high grade aminonaphthol sulfonic acid, solution is complete and rapid; with less suitable grades, it may be necessary to bring the acid into solution by the addition of a few more drops of the sulfite solution, carefully avoiding an excess. Preserve in a brown bottle away from light—stability approximately two weeks. The fresh solution should be almost colorless—on aging it acquires a yellow tinge; discard when the reagent blank shows an appreciable increase.
   (a) Sodium bisulfite, 15% aqueous; dissolve 15 gm sodium bisulfite ($NaHSO_3$—Merck, Reagent) in distilled water and dilute to 100 ml.
   (b) Sodium sulfite, 20% aqueous; dissolve 20 gm anhydrous sodium sulfite (Na_2SO_3—Baker & Adamson, A.C.S.) in distilled water and dilute to 100 ml.

Technic

Note: If the analysis is to be carried out on an aliquot of the trichloracetic acid centrifugate (or filtrate) as reserved in the inorganic phosphorus determination (Method P-36, Step 4), omit the following Steps 1–4 inclusive.

1. In a 15 ml glass-stoppered centrifuge tube (sample) place
   9 ml 5% trichloracetic acid (1)
   1 ml sample from an Ostwald-Folin pipette, added drop by drop, with constant agitation of the tube.
2. In another 15 ml glass-stoppered centrifuge tube (reagent blank) place
   9 ml 5% trichloracetic acid (1)
   1 ml distilled water.
3. Stopper both tubes and mix thoroughly.
4. Allow to stand for 5 minutes and centrifuge at 1500 rpm for 3 minutes (filtrates may be employed but the contents of *both* tubes must be filtered through dry 7 cm double acid-washed, calcium and phosphorus-free papers into dry tubes). RESERVE PORTION FOR INORGANIC PHOSPHORUS ANALYSIS (Method P-36).
5. In a Hoffman-Fister calcium tube place 3 ml supernatant fluid (or filtrate) of sample.
6. In another Hoffman-Fister calcium tube place 3 ml supernatant fluid (or filtrate) of reagent blank.
7. To each tube add 0.3 ml 6.25 N sodium hydroxide (2).
8. Mix and allow to stand for 5 minutes.
9. Add 0.3 ml 1% trisodium phosphate (3).
10. Mix with rotatory movement of the tubes and allow to stand for one hour.
11. Centrifuge at 2000 rpm for 5 minutes.
12. Decant and discard the supernatant fluids and drain the tubes by inverting on filter papers for 5 minutes; wipe off mouths of tubes with clean tissues.
13. Add 2 ml alkaline alcohol wash solution (4) and stir up the precipitates with platinum-tipped stirring rods, washing off the rods as they are being removed from the tubes with a fine stream of alkaline alcohol from an all-glass wash bottle, rinsing down the sides of the tubes at the same time.
14. Centrifuge at 2000 rpm for 3 minutes.
15. Decant and discard the supernatant fluids and drain as above for 3 minutes.
16. Repeat Steps 13–15 inclusive but drain for 20 minutes, carefully wiping off the lips of the tubes until all the alcohol is removed.
17. Add 3 ml 5% trichloracetic acid (1) and stir with *another* set of platinum-tipped stirring rods until the precipitates have dissolved.
18. Add 0.5 ml molybdate reagent II (5) and mix.
19. Add 0.5 ml aminonaphthol sulfonic acid reagent (6) and mix.
20. Dilute to 8 ml with distilled water; stopper with clean rubber stoppers and mix thoroughly by inversion.
21. Allow to stand in the dark for 10 minutes.
22. Measure the transmittancy of the sample against the reagent blank set at 100% transmission at a wave length of **660** mμ and multiply the value from the calibration table for inorganic phosphorus (Method P-36) by 1.94 to obtain the concentration of calcium in mg per 100 ml sample. If a calibration table has been prepared for calcium, the concentration is obtained directly.

CALIBRATION PROCEDURE

Note: The following calibration procedures are optional—the values in the calibration table for inorganic phosphorus (Method P-36), when multiplied by 1.94, are equivalent to the concentration of calcium in mg per 100 ml sample. Two procedures are described—(A) which affords a rapid means of accurately constructing a calibration curve and table and (B) which is recommended as a check on the analyst's technical skill. With either procedure Calibration Curve C-10 should be obtained.

Procedure (A)

Special Apparatus

Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.

Graduated test tubes—10 ml in 0.1 ml; the 8 ml graduation must be accurately calibrated.

Reagents

1. **Trichloracetic Acid, 5% Aqueous.** Same as for Test Procedure.
5. **Molybdate Reagent II (Fiske-SubbaRow).** Same as for Test Procedure.
6. **Aminonaphthol Sulfonic Acid Reagent (Fiske-SubbaRow).** Same as for Test Procedure.
7. **Calcium Standards**
    (a) Calcium stock standard (1 ml equivalent to 1 mg calcium). Dry recrystallized monopotassium phosphate (KH_2PO_4—Reagent Chemicals, Special) at 100° C. for one hour and cool in desiccator to constant weight; transfer 226.5 mg to a 100 ml volumetric flask; add 50 ml distilled water and shake until dissolved; dilute to volume with same. Preserve with 5 ml anhydrous chloroform.
    (b) Dilute standard (1 ml equivalent to 0.02 mg calcium). In a 100 ml volumetric flask place 2 ml stock standard (7a) and dilute to volume with 5% trichloracetic acid (1). Prepare just before use.
    (c) Calibration standards. In a series of six accurately calibrated 10 ml volumetric flasks place respectively 0, 2, 4, 6, 8 and 10 ml of the dilute standard (7b) and dilute to volume with 5% trichloracetic acid (1). These standards represent concentrations of calcium equivalent to 0, 4, 8, 12, 16 and 20 mg per 100 ml sample. Prepare just before use.

Technic

1. Transfer 3 ml of each calibration standard (7c) to a correspondingly marked accurately graduated 10 ml test tube.
2. Proceed exactly as outlined in the Test Procedure, Steps 18–21 inclusive.
3. Set the zero concentration standard at 100% transmission at a wave length of 660 mμ and record the transmittancy of the 4, 8, 12, 16 and 20 mg per cent standards. The transmittancy of the 20 mg standard should be approximately 24%.
4. Plot the observed values on semi-log graph paper—the points fall on a straight line which passes through the origin (Calibration Curve C-10).
5. Prepare a calibration table from the T-C values obtained from the plotted curve.

Procedure (B)

Special Apparatus

Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.
Calcium tubes—Hoffman-Fister, accurately calibrated at the 8 ml graduation.
Stirring rods, glass, platinum-tipped.
Wash bottles, all-glass, Pyrex, 125 ml.

Reagents

1. **Trichloracetic Acid, 5% Aqueous.** Same as for Test Procedure.
2. **Sodium Hydroxide, Volumetric 6.25 N.** Same as for Test Procedure.
3. **Sodium Phosphate, Tribasic, 1% Aqueous.** Same as for Test Procedure.
4. **Alkaline Alcohol Wash Solution (Roe-Kahn).** Same as for Test Procedure.
5. **Molybdate Reagent II (Fiske-SubbaRow).** Same as for Test Procedure.
6. **Aminonaphthol Sulfonic Acid Reagent (Fiske-SubbaRow).** Same as for Test Procedure.
8. **Calcium Standards**
    (a) Calcium stock standard (1 ml contains 1 mg calcium). In a 100 ml volumetric flask place 249.6 mg finely pulverized calcium carbonate ($CaCO_3$—Baker & Adamson, Iceland Spar, Crystal (Calcite) for Standardization); add 10 ml distilled water and 5 ml concentrated trichloracetic acid (1a); shake until dissolved and dilute to volume with distilled water.
    (b) Dilute standard (1 ml contains 0.02 mg calcium). In a 100 ml volumetric flask place 2 ml stock standard (8a) and dilute to volume with 5% trichloracetic acid (1). Prepare just before use.
    (c) Calibration standards. In a series of six accurately calibrated 10 ml volumetric flasks place respectively 0, 2, 4, 6, 8 and 10 ml of the dilute standard (8b) and dilute to volume with 5% trichloracetic acid (1). These standards represent concentrations of calcium equivalent to 0, 4, 8, 12, 16 and 20 mg per 100 ml sample. Prepare just before use.

Technic

1. Transfer 3 ml of each calibration standard (8c) to a correspondingly marked Hoffman-Fister calcium tube.
2. Proceed exactly as outlined in the Test Procedure, Steps 7–21 inclusive.
3. Set the zero concentration standard at 100% transmission at a wave length of 660 mμ and record the transmittancy of the 4, 8, 12, 16 and 20 mg per cent standards. The transmittancy of the 20 mg standard should be approximately 24%.
4. Plot the observed values on semi-log graph paper—the points fall on a straight line which passes through the origin (Calibration Curve C-10).
5. Prepare a calibration table from the T-C values obtained from the plotted curve.

Method C-10b

DETERMINATION IN URINE

PRINCIPLE

Calcium is precipitated from the protein and chromogen-free urine as the oxalate, which is then dissolved in trichloracetic acid. The calcium is reprecipitated as the phosphate and the phosphorus determined as outlined for blood.

SAMPLE

Aliquot of a 24-hour specimen. For three days prior to the collection of the sample, the subject must be on a low calcium and neutral ash diet.

TEST PROCEDURE

Special Apparatus

Ostwald-Folin pipettes accurately calibrated "to contain" 5 ml.
Graduated centrifuge tubes—15 ml.
Capillary droppers delivering 40 drops per ml.
Calcium tubes—Hoffman-Fister, accurately calibrated at the 8 ml graduation.
Stirring rods, glass, platinum-tipped.
Wash bottles, all-glass, Pyrex, 125 ml.
Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.

Note: See remarks under special apparatus for blood.

Reagents

1. **Trichloracetic Acid, 5% Aqueous.** Same as for blood.
2. **Sodium Hydroxide, Volumetric 6.25 N.** Same as for blood.
3. **Sodium Phosphate, Tribasic, 1% Aqueous.** Same as for blood.
4. **Alkaline Alcohol Wash Solution (Roe-Kahn).** Same as for blood.
5. **Molybdate Reagent II (Fiske-SubbaRow).** Same as for blood.
6. **Aminonaphthol Sulfonic Acid Reagent (Fiske-SubbaRow).** Same as for blood.
9. **Norit A, Acid-Washed (Andes-Myers).** In a 1000 ml beaker place 100 gm norit A (Pfanstiehl) and add 100 ml 0.5 N hydrochloric acid (a); heat to boiling and filter while hot on a Büchner funnel with suction; wash the norit on the filter with successive portions of distilled water until the washings are chloride-free; remove from filter and dry in a hot air oven at 120° C. for 24 hours—do not heat to high temperatures.
   (a) Hydrochloric acid, 0.5 N; dilute 5 ml concentrated hydrochloric acid (HCl—Baker & Adamson, A.C.S.) to 100 ml with distilled water and mix.
10. **Sodium Acetate, 20% Aqueous.** Dissolve 20 gm crystalline sodium acetate ($NaC_2H_3O_2 \cdot 3H_2O$—Baker & Adamson, A.C.S.) in distilled water and dilute to 100 ml; filter.
11. **Bromcresol Green Indicator Solution, 0.016% Aqueous.** In a 250 ml volumetric flask place 40.8 mg of the water-soluble sodium salt of bromcresol green ($C_{21}H_{14}Br_4O_5S$—LaMotte); dissolve in distilled water and dilute to volume. Preserve in resistant-glass bottle.
12. **Ammonium Oxalate, 0.1 Molar.** Dissolve 1.421 gm crystalline ammonium oxalate ($(NH_4)_2C_2O_4 \cdot H_2O$—Merck, A.C.S.) in distilled water and dilute to 100 ml. Add a crystal of thymol and preserve in refrigerator.
13. **Ammonium Hydroxide, 10% by Volume.** Dilute 10 ml concentrated ammonium hydroxide (NH_4OH—Baker & Adamson, A.C.S.) to 100 ml with distilled water. Preserve in resistant-glass bottle.
14. **Acetic Acid, 10% by Volume.** Dilute 10 ml glacial acetic acid (CH_3COOH—Baker & Adamson, A.C.S.) to 100 ml with distilled water.
15. **Ammoniacal Wash Solution (Wang).** Add 10 ml concentrated ammonium hydroxide (NH_4OH—Baker & Adamson, A.C.S.) to 490 ml alcohol-ether solution (a) and mix. Preserve and use in an all-glass Pyrex wash bottle.
   (a) Alcohol-ether solution; mix 200 ml ether ($(C_2H_5)_2O$—Mallinckrodt, A.C.S.) with 200 ml 95% ethyl alcohol (C_2H_5OH—U.S. Industrial Chemicals, U.S.P.) and add 200 ml distilled water; mix well. Preserve in a glass-stoppered bottle.
16. **Trichloracetic Acid, 4.5% Aqueous.** Dilute 90 ml 5% trichloracetic acid (1) to 100 ml with distilled water. Prepare fresh weekly.

Technic

1. In a 50 ml Erlenmeyer flask (sample) place
   15 ml distilled water
   5 ml thoroughly mixed unfiltered urine from an Ostwald-Folin pipette, thoroughly rinsing out the pipette by drawing in and expelling some of the mixture several times
   5 ml 5% trichloracetic acid (1).
2. In another 50 ml Erlenmeyer flask (reagent blank) place
   20 ml distilled water
   5 ml 5% trichloracetic acid (1).
3. To each flask add 100 mg acid-washed norit A (9), stopper and shake vigorously for 30 seconds.
4. Allow to stand for 20 minutes.
5. Filter the contents of each flask through Schleicher and Schuell #576 papers into correspondingly marked test tubes.

6. Transfer 2.5 ml of each filtrate to a correspondingly marked 15 ml graduated centrifuge tube and add 2.5 ml distilled water.
7. Add 1 ml 20% sodium acetate (10) and 5 drops bromcresol green indicator solution (11)—the color of the solution is yellow.
8. Add 1 ml 0.1 M ammonium oxalate (12), allowing the solution to fall directly into the fluid in the tubes and mix with thin glass rods—allow the rods to remain in the tubes.
9. Add sufficient 10% ammonium hydroxide (13), drop by drop from a capillary-tipped dropper, until the color becomes distinctly green (pH 5.0)—if an excess of alkali is added and the color becomes too blue, adjust to the green color by adding 10% acetic acid (14), drop by drop.
10. Remove rods from tubes, rinsing with about 2 ml distilled water.
11. Allow to stand for 3 hours (preferably over night) in the refrigerator.
12. Centrifuge at 2000 rpm for 15 minutes.
13. Carefully decant and discard the supernatant fluids and drain the tubes by inverting on filter papers for 5 minutes; wipe off mouths of tubes with clean tissues.
14. Add 3 ml ammoniacal wash solution (15) and stir up the precipitates with platinum-tipped stirring rods, washing off the rods as they are being removed from the tubes with a fine stream of wash solution from an all-glass wash bottle, rinsing down the sides of the tubes at the same time.
15. Centrifuge at 2000 rpm for 15 minutes.
16. Decant and discard the supernatant fluids and drain as above for 3 minutes.
17. Repeat Steps 14–16 inclusive but drain for 20 minutes, carefully wiping off the lips of the tubes until all the wash solution is removed.
18. Add 5 ml 4.5% trichloracetic acid (16) and dissolve the precipitates, using gentle heat if necessary.
19. Cool to room temperature and add sufficient 4.5% trichloracetic acid (16) to bring the volume to 10 ml.
20. In a Hoffman-Fister calcium tube place 3 ml of the fluid from the sample tube.
21. In another Hoffman-Fister calcium tube place 3 ml of the fluid from the reagent blank tube.
22. Proceed exactly as outlined in the Test Procedure for blood, Steps 7–21 inclusive.
23. Measure the transmittancy of the sample against the reagent blank set at 100% transmission at a wave length of **660** mμ and multiply the value from the calibration table for inorganic phosphorus (Method P-36) by 3.88 to obtain the concentration of calcium in mg per 100 ml urine. If a calibration table has been prepared for calcium in blood, multiply the value obtained by 2.

Calculation

$$\frac{\text{Volume of 24-hour specimen}}{100} \times \text{Calcium in mg per 100 ml} = \text{Mg calcium excreted in 24 hours}$$

Method C-10c

DETERMINATION IN FECES

PRINCIPLE

The organic matter in the dried and pulverized feces is oxidized by means of a nitric-perchloric acid digestion mixture. The calcium is then precipitated as the oxalate, dissolved in trichloracetic acid and reprecipitated as the phosphate. The phosphorus is determined as outlined for blood.

SAMPLE

Entire 24-hour specimen. For three days prior to the collection of the sample, the subject must be on a low calcium and neutral ash diet. Time intervals may be definitely determined by the administration of carmine capsules.

TEST PROCEDURE

Special Apparatus

Large porcelain evaporating dish.
Micro burner.
Graduated centrifuge tubes—15 ml.
Capillary droppers delivering 40 drops per ml.
Calcium tubes—Hoffman-Fister, accurately calibrated at the 8 ml graduation.
Stirring rods, glass, platinum-tipped.
Wash bottles, all-glass, Pyrex, 125 ml.
Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.

Note: See remarks under special apparatus for blood.

Reagents

1. **Trichloracetic Acid, 5% Aqueous.** Same as for blood.
2. **Sodium Hydroxide, Volumetric 6.25 N.** Same as for blood.
3. **Sodium Phosphate, Tribasic, 1% Aqueous.** Same as for blood.
4. **Alkaline Alcohol Wash Solution (Roe-Kahn).** Same as for blood.

5. **Molybdate Reagent II (Fiske-SubbaRow).** Same as for blood.
6. **Aminonaphthol Sulfonic Acid Reagent (Fiske-SubbaRow).** Same as for blood.
10. **Sodium Acetate, 20% Aqueous.** Same as for urine.
11. **Bromcresol Green Indicator Solution, 0.016% Aqueous.** Same as for urine.
12. **Ammonium Oxalate, 0.1 Molar.** Same as for urine.
13. **Ammonium Hydroxide, 10% by Volume.** Same as for urine.
14. **Acetic Acid, 10% by Volume.** Same as for urine.
15. **Ammoniacal Wash Solution (Wang).** Same as for urine.
16. **Trichloracetic Acid, 4.5% Aqueous.** Same as for urine.
17. **Nitric-Perchloric Acid Digestion Mixture (Wang).** In a 30 ml Erlenmeyer flask place 5 ml fuming nitric acid (HNO_3—Baker & Adamson, A.C.S.); while constantly agitating the flask, slowly add 2.5 ml 60% perchloric acid ($HClO_4$—G. Frederick Smith, Vacuum Distilled) and 5 ml distilled water; mix. Do not prepare a larger quantity than required for immediate use—discard any excess.

Technic

Note: For concentrations of calcium in excess of 20 mg per gm, dilute to 50 ml in Step 11 and multiply the values from the calibration table for inorganic phosphorus (Method P-36) by 3.88 to obtain the concentration of calcium in mg per gm dried feces; or multiply the values from the calibration table for blood by 2 to obtain the concentration of calcium in mg per gm dried feces.

1. In a large weighed porcelain evaporating dish place the entire 24-hour specimen.
2. Dry on a steam bath for 6 hours and then in a hot air oven (at a temperature not over 100° C.) for 24 hours or until thoroughly dry.
3. Cool to room temperature and weigh evaporating dish and contents—record weight of sample.
4. Transfer about 1 gm of the dried feces to a mortar and pulverize.
5. In a 125 ml Pyrex Kjeldahl flask place exactly 50 mg of the pulverized feces.
6. Add 4 ml nitric-perchloric acid digestion mixture (17), rinsing off the weighing crystal into the flask at the same time.

Note: Steps 7–10 inclusive should be carried out in a fume hood.

7. Heat flask over a micro burner with constant shaking—during this operation it is advisable that the worker be protected by a shatter-proof shield.
8. When the fluid darkens and white fumes appear, remove the flask from the burner and add another 4 ml portion of the digestion mixture.
9. Continue heating until the digest clears or is only slightly colored.
10. Allow to cool to room temperature.
11. Quantitatively transfer the contents of the flask to a 25 ml volumetric flask and dilute to volume with distilled water.
12. Transfer 5 ml to a 15 ml graduated centrifuge tube.
13. Proceed exactly as outlined in the Test Procedure for *urine*, Steps 7–19 inclusive.
14. In a Hoffman-Fister calcium tube (sample) place 3 ml of the trichloracetic acid solution of the precipitate.
15. In another Hoffman-Fister calcium tube (reagent blank) place 3 ml 4.5% trichloracetic acid (16).
16. Proceed exactly as outlined in the Test Procedure for *blood*, Steps 7–21 inclusive.
17. Measure the transmittancy of the sample against the reagent blank set at 100% transmission at a wave length of **660** mμ and multiply the value from the calibration table for inorganic phosphorus (Method P-36) by 1.94 to obtain the concentration of calcium in mg per gm dried feces. If a calibration table has been prepared for calcium in blood, the concentration is obtained directly and expressed as mg per gm dried feces.

Calculation

Weight of 24-hour specimen in gm (from Step 3) × Calcium in mg per gm = Mg calcium excreted in 24 hours

PROCEDURE FOR USE WITH LINEAR-SCALE PHOTOELECTRIC COLORIMETERS (SHEARD-SANFORD TYPE)

TEST PROCEDURE

Exactly as outlined; set the reagent blank at 100 and use a red filter.

CALIBRATION PROCEDURE

Exactly as outlined—use a red filter; plot the observed readings on semi-log graph paper—the points fall on a straight line passing through the origin.

PROCEDURE FOR USE WITH LOGARITHMIC-SCALE PHOTOELECTRIC COLORIMETERS (KLETT-SUMMERSON TYPE)

TEST PROCEDURE

Exactly as outlined; set the reagent blank at 0 and use a red filter.

CALIBRATION PROCEDURE

Exactly as outlined—use a red filter; plot the observed readings on linear graph paper—the points fall on a straight line passing through the origin.

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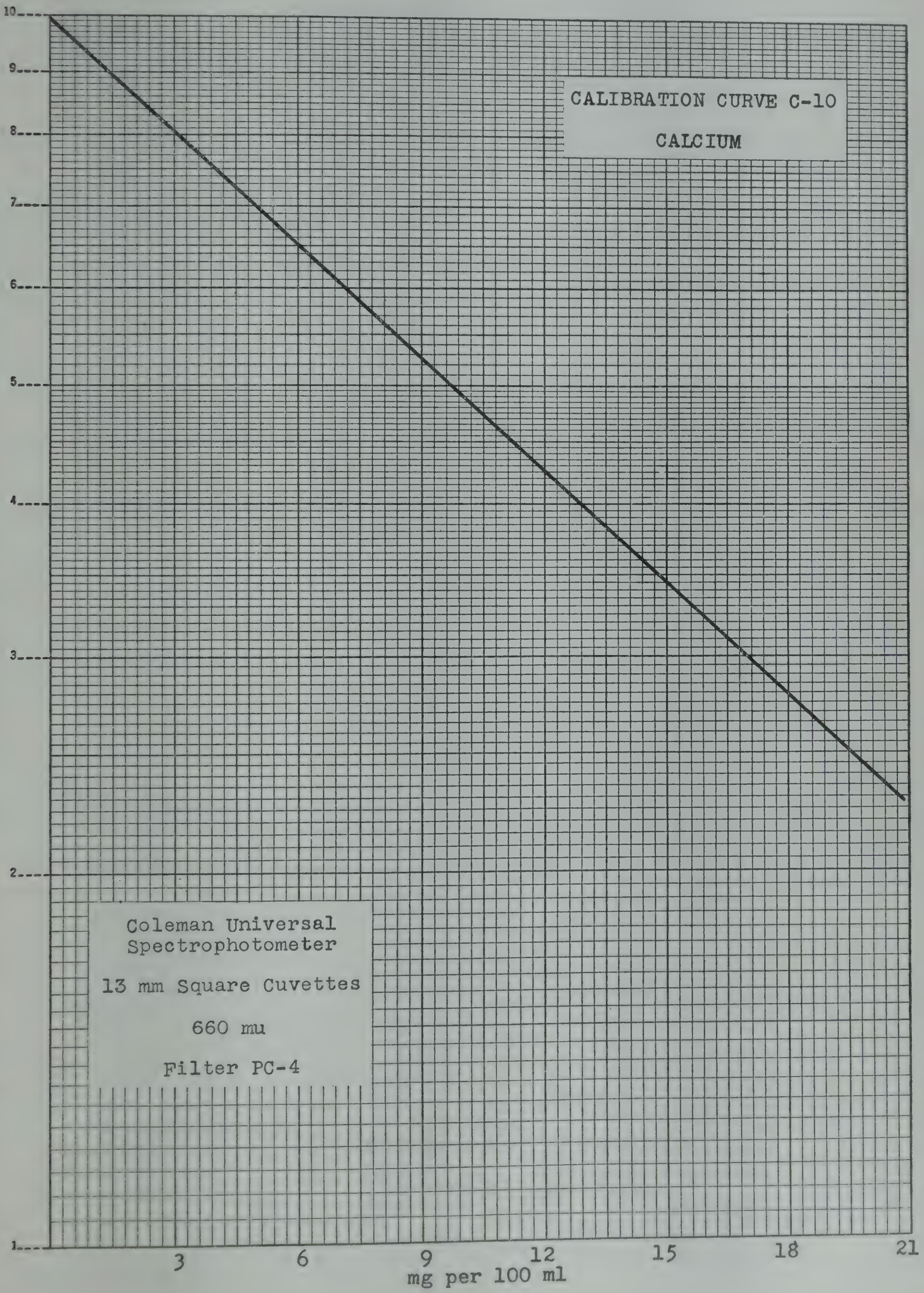
CALIBRATION CURVE C-10
CALCIUM
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660 mu
Filter PC-4
10
9
8
7
6
5
4
3
2
1
3
6
9
12
15
18
21
mg per 100 ml

Meter Reading	0	1	2	3	4	5	6	7	8	9
0										
10										
20										
30										
40										
50										
60										
70										
80										
90										

CARBON DIOXIDE CAPACITY

A spectrophotometric adaptation based on the method of Exton, Schattner and Rose (1941)

PRINCIPLE

Blood plasma is saturated with alveolar carbon dioxide in the absence of atmospheric air. Upon treatment with barium hydroxide, the carbon dioxide is precipitated as barium carbonate and the latter converted to barium chloride which, in turn, is treated with ammonium chromate to form barium chromate. Free chromic acid is liberated by means of hydrochloric acid and the chromium content determined by its rose-red reaction with diphenylcarbazide. Thus, indirectly, the original carbon dioxide capacity of the plasma is ascertained.

DETERMINATION IN PLASMA

SAMPLE

Plasma from blood withdrawn at any time of the day but only after an adequate rest period to clear the blood of any lactic acid which may have been formed by previous activity. If the carbon dioxide capacity only is to be determined, collection of the sample is made as outlined. When other determinations are required, the blood may be part of an oxalated venous sample provided that adequate precautions are taken (such as, collection under oil and the use of pipettes with rubber bulbs) to avoid exposure to atmospheric and especially expired air—for a more detailed description see collection of sample under oxygen capacity (Method O-10).

COLLECTION: Connect a collecting cup (Fig. C-15a) to a small oxalated centrifuge tube (Fig. C-15b); fill tube and cup to about one inch below the edge of the cup with mineral oil (Reagent #2). Puncture the finger, wipe off the first drop of blood, and quickly submerge the finger tip under the oil in the cup; as soon as the volume of blood reaches or exceeds the 0.5 ml mark on the tube, remove the finger from the cup. Mix blood and anticoagulant thoroughly by stirring with a thin glass rod; carefully pour the oil out of the cup and the upper third of the centrifuge tube; detach the cup and place the centrifuge tube in the refrigerator. Separate the plasma from the red blood cells as soon as possible (within thirty minutes) by centrifuging at 1800 rpm for 5 minutes. Plasma samples, under oil, may be preserved in refrigerator for several days.

TEST PROCEDURE

Special Apparatus

A single determination requires the following:
Collection cup with ground glass joint (Fig. C-15a).
Centrifuge tube, 5 ml, graduated at 0.5 ml, with ground glass joint to fit the collection cup (Fig. C-15b). Prepare this tube before use by adding 0.2 ml double oxalate anticoagulant solution (Reagent #1) and evaporating to dryness at low heat so that the dry mixture adheres to the walls of the tube below the 0.5 ml mark.
Capillary pipette with rubber bulb.
Saturation assembly (Fig. C-15c).
Centrifuge tube, Pyrex, 15 ml.
Pipette with rubber bulb, 1 ml.
Ostwald-Van Slyke pipette, accurately calibrated "to deliver" 0.1 ml.
Pipette with rubber bulb, 0.2 ml.
Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.

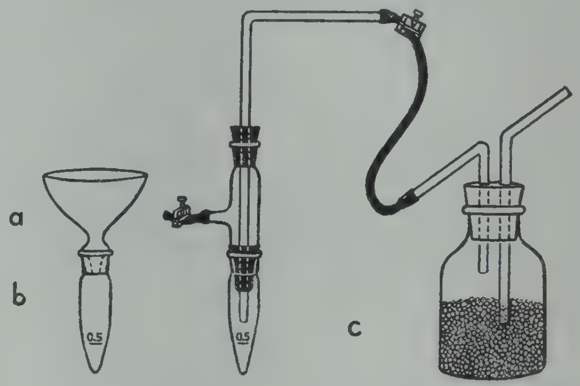

Fig. C-15.

Reagents

1. **Double Oxalate Anticoagulant Solution (Heller-Paul).** Dissolve 6 gm crystalline ammonium oxalate ($(NH_4)_2C_2O_4 \cdot H_2O$—Baker & Adamson, A.C.S.) and 4 gm neutral crystalline potassium oxalate ($K_2C_2O_4 \cdot H_2O$—J. T. Baker, CP Analyzed) in distilled water and dilute to 1000 ml.
2. **Mineral Oil** (Marcol GX—Standard Oil Co. of U.S.).
3. **Water, Distilled, Carbon Dioxide-Free.** In a 500 ml flask place 300 ml distilled water; boil for 5 minutes; insert rubber stopper with soda-lime guard and allow to cool to room temperature. This must be used where indicated.
4. **Barium Hydroxide, Ammoniacal (Exton-Schattner-Rose).** In a 100 ml volumetric flask place 2.5 gm crystalline barium hydroxide ($Ba(OH)_2 \cdot 8H_2O$—J. T. Baker, CP Analyzed) and dissolve in 40 ml freshly boiled carbon dioxide-free distilled water (3); add 50 ml ammonium hydroxide (NH_4OH—Baker & Adamson, A.C.S.) and mix; dilute to volume with carbon dioxide-free distilled water and filter. Preserve under a 2 cm layer of mineral oil (2) in a resistant-glass

bottle. Use only the clear supernatant fluid; do not disturb any white sediment which may form.

5. **Celite** (Johns-Manville Co.).
6. **Hydrochloric Acid, Volumetric 4.5 N.** Add 450 ml concentrated hydrochloric acid (HCl—Baker & Adamson, A.C.S.) to 550 ml carbon dioxide-free distilled water (3) and mix; allow to cool; dilute 20 ml of this approximately 4.5 N solution to 100 ml; titrate against standard 1 N alkali using phenolphthalein as indicator; adjust accordingly and re-titrate—20 ml of a 1:5 dilution of 4.5 N acid require 18 ml of 1 N alkali.
7. **Ammonium Chromate, Ammoniacal (Exton-Schattner-Rose).** In a 100 ml volumetric flask place 250 mg ammonium chromate ($(NH_4)_2CrO_4$—J. T. Baker, CP Analyzed) and dissolve in 40 ml distilled water; add 50 ml ammonium hydroxide (NH_4OH—Baker & Adamson, A.C.S.) and mix; dilute to volume with distilled water. Preserve in resistant-glass bottle.
8. **Hydrochloric Acid, Concentrated** (HCl—Baker & Adamson, A.C.S.).
9. **Diphenylcarbazide, 1% Alcoholic.** Dissolve 1 gm s-diphenylcarbazide ($(C_6H_5)_2(NH)_4CO$—Eastman #618) in 100 ml 95% ethyl alcohol (C_2H_5OH—U.S. Industrial Chemicals, U.S.P.). Preserve in brown glass bottle away from direct light. The reagent has a pale yellowish color; discard when a red tint develops.

Technic

SATURATION OF PLASMA WITH CARBON DIOXIDE: By means of a capillary pipette with rubber bulb, transfer the oil-free plasma from the original 5 ml centrifuge tube to the similar 5 ml centrifuge tube in the saturation assembly (Fig. C-15c). The plasma is saturated with carbon dioxide at normal alveolar tension by means of the saturation assembly (Fig. C-15c) without inspiring more deeply than normal and expiring as quickly and completely as possible into the inlet mouthpiece in the jar of glass beads. The first blow through the assembly is made with both clamps open. The clamps are closed between the third and fourth blows. The plasma is spread in a thin layer over the inside of the tube by gently rotating the tube in a semi-horizontal position between blows. In the above operation, extreme care must be taken to avoid diluting the alveolar carbon dioxide with atmospheric air.

1. In a 15 ml Pyrex centrifuge tube place 1 ml boiling carbon dioxide-free distilled water (3) by means of a pipette with a rubber bulb—do not aspirate by mouth.
2. Stopper and cool under running tap water.
3. Add 0.1 ml carbon dioxide saturated plasma from an Ostwald-Van Slyke pipette and mix thoroughly.
4. Add 0.2 ml ammoniacal barium hydroxide (4) by means of a pipette with a rubber bulb, wiping off any adherent oil.
5. Allow to stand for 5 minutes.
6. Add 10 ml carbon dioxide-free distilled water (3).
7. Add 0.5 mg celite (5) and mix thoroughly by gently inverting the tube two or three times.
8. Centrifuge at low speed (about 800 rpm) for 2 minutes and then at high speed (about 1800 rpm) for 3 minutes—precipitate must be in the tip of the tube—not on the walls.
9. Decant supernatant fluid and drain the tube thoroughly on absorbent paper for 5 minutes—wipe off any liquid around the mouth of the still inverted tube.
10. Add 0.5 ml 4.5 N hydrochloric acid (6) and mix.
11. Place the tube in boiling water for 30 seconds.
12. Cool at once under running tap water.
13. Add 1 ml ammoniacal ammonium chromate (7) and mix.
14. Allow to stand for 5 minutes.
15. Add 10 ml distilled water.
16. Repeat Steps 8–9 inclusive.
17. Add 10 ml distilled water and 1 ml concentrated hydrochloric acid (8), stirring with a thin glass rod until the precipitate is completely dissolved; drain the rod into the tube.
18. Transfer the contents of the tube quantitatively to a 100 ml volumetric flask (sample).
19. In another 100 ml volumetric flask (reagent blank) place
    10 ml distilled water
    1 ml concentrated hydrochloric acid (8).
20. To each flask add approximately 75 ml distilled water.
21. Add 0.5 ml 1% diphenylcarbazide (9).
22. Dilute to volume with distilled water and mix.
23. Measure the transmittancy of the sample against the reagent blank set at 100% transmission at a wave length of **480** mμ and obtain the concentration of carbon dioxide from the calibration table.

CALIBRATION PROCEDURE

Special Apparatus

Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.

Reagents

8. **Hydrochloric Acid, Concentrated.** Same as for Test Procedure.
9. **Diphenylcarbazide, 1% Alcoholic.** Same as for Test Procedure.
10. **Carbon Dioxide Capacity Standards**
    (a) Carbon dioxide capacity stock standard (1 ml contains 0.0882 mg potassium chromate). Dry finely pulverized crystalline potassium chromate (K_2CrO_4—Reagent Chemicals, Special) in a hot air oven at 110° C. for one

hour; cool; transfer 88.2 mg to a 1000 ml volumetric flask; dissolve in distilled water and dilute to volume.

(b) Calibration standards. In a series of five accurately calibrated 100 ml volumetric flasks place respectively 0, 2.5, 5, 7.5 and 10 ml stock standard (10a); add approximately 80 ml distilled water; add 1 ml concentrated hydrochloric acid (8) and 0.5 ml 1% diphenylcarbazide (9); dilute to volume with distilled water and mix. These standards are equivalent in color to 0, 25, 50, 75 and 100 volumes per cent carbon dioxide respectively. Prepare just before use and proceed immediately as follows.

Technic

1. Transfer a suitable portion of each calibration standard (10b) to a correspondingly marked cuvette.
2. Set the zero concentration standard at 100% transmission at a wave length of **480** mμ and record the transmittancy of the 25, 50, 75 and 100% standards. The transmittancy of the 100% standard should be approximately 24%.
3. Plot the observed values on semi-log graph paper—the points fall on a smooth curve which passes through the origin (Calibration Curve C-15).
4. Prepare a calibration table from the T-C values obtained from the plotted curve.

PROCEDURE FOR USE WITH LINEAR-SCALE PHOTOELECTRIC COLORIMETERS (SHEARD-SANFORD TYPE)

TEST PROCEDURE

Exactly as outlined; set the reagent blank at 100 and use a blue filter.

CALIBRATION PROCEDURE

Exactly as outlined—use a blue filter; plot the observed readings on semi-log graph paper—the points fall on a smooth curve passing through the origin.

PROCEDURE FOR USE WITH LOGARITHMIC-SCALE PHOTOELECTRIC COLORIMETERS (KLETT-SUMMERSON TYPE)

TEST PROCEDURE

Exactly as outlined; set the reagent blank at 0 and use a blue filter.

CALIBRATION PROCEDURE

Exactly as outlined—use a blue filter; plot the observed readings on linear graph paper—the points fall on a smooth curve passing through the origin.

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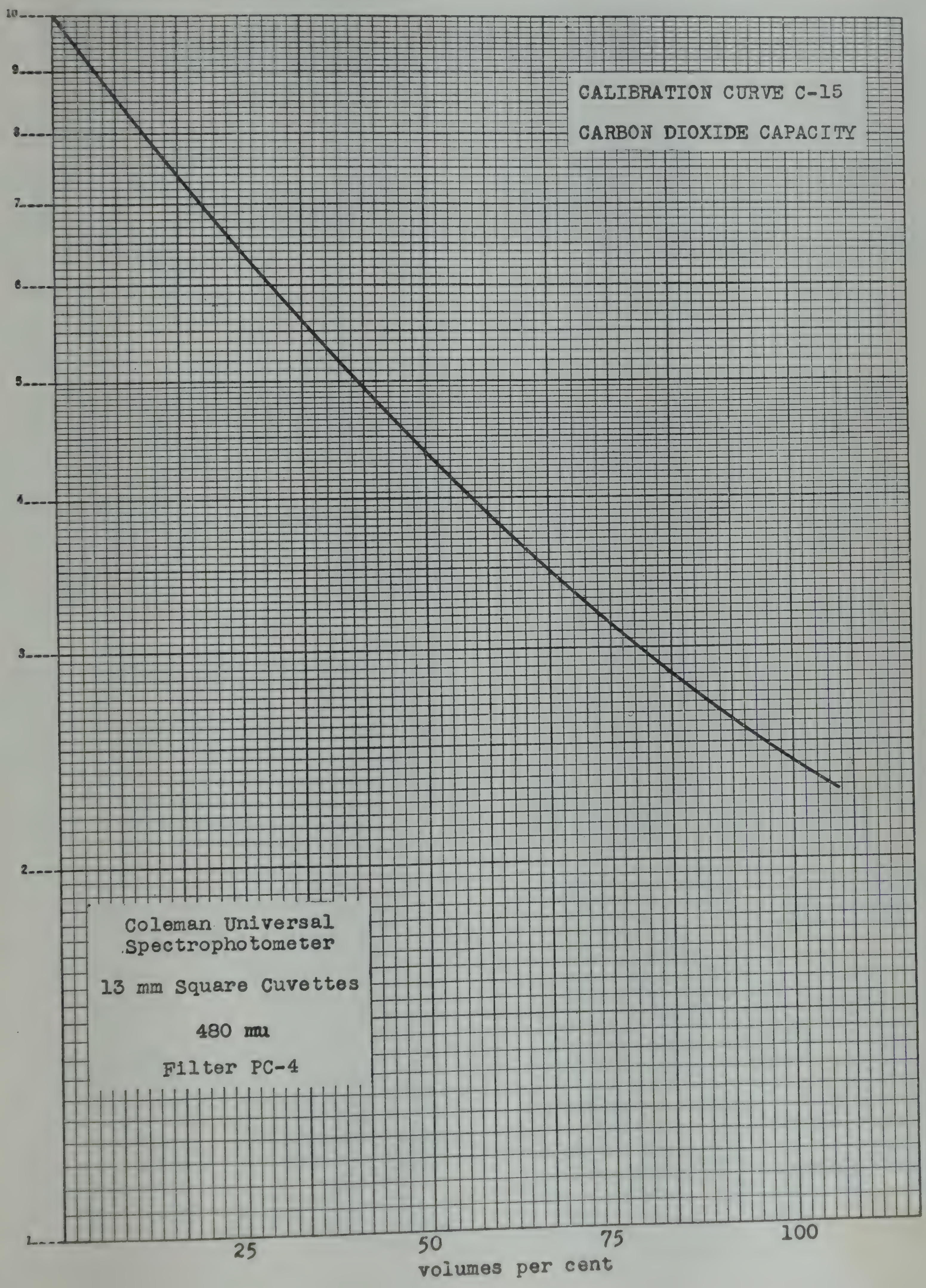
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5
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2
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25
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100
volumes per cent

Meter Reading	0	1	2	3	4	5	6	7	8	9
0										
10										
20										
30										
40										
50										
60										
70										
80										
90										

CARBON DIOXIDE CONTENT

A spectrophotometric adaptation based on the methods and modifications of Stadie and Van Slyke (1920) and Exton, Schattner and Rose (1941)

PRINCIPLE

Carbon dioxide is precipitated as barium carbonate from blood plasma without any previous treatment. The analysis is then carried out as described for carbon dioxide capacity (Method C-15), the same analytical principles being involved.

DETERMINATION IN PLASMA

SAMPLE

Plasma from blood withdrawn at any time of the day but only after an adequate rest period to clear the blood of any lactic acid which may have been formed by previous activity. If the carbon dioxide content only is to be determined, collection of the sample is made as outlined. When other determinations are required, the blood may be part of an oxalated venous sample provided that adequate precautions are taken (such as, collection under oil and the use of pipettes with rubber bulbs) to avoid exposure to atmospheric and especially expired air—for a more detailed description see collection of sample under oxygen capacity (Method O-10).

Collection: Connect a collecting cup (Fig. C-15a) to a small oxalated centrifuge tube (Fig. C-15b); fill tube and cup to about one inch below the edge of the cup with mineral oil (Reagent #2). Puncture the finger, wipe off the first drop of blood, and quickly submerge the finger tip under the oil in the cup; as soon as the volume of blood reaches or exceeds the 0.5 ml mark on the tube, remove the finger from the cup. Mix blood and anticoagulant thoroughly by stirring with a thin glass rod; carefully pour the oil out of the cup and the upper third of the centrifuge tube; detach the cup and place the centrifuge tube in the refrigerator. Separate the plasma from the red blood cells as soon as possible (within thirty minutes) by centrifuging at 1800 rpm for 5 minutes. Plasma samples, under oil, may be preserved in refrigerator for several days.

TEST PROCEDURE

Special Apparatus

A single determination requires the following:

Collection cup with ground glass joint (Fig. C-15a).

Centrifuge tube, 5 ml, graduated at 0.5 ml, with ground glass joint to fit the collection cup (Fig. C-15b). Prepare this tube before use by adding 0.2 ml double oxalate anticoagulant solution (Reagent #1) and evaporating to dryness at low heat so that the dry mixture adheres to the walls of the tube below the 0.5 ml mark.

Capillary pipette with rubber bulb.

Centrifuge tube, Pyrex, 15 ml.

Pipette with rubber bulb, 1 ml.

Ostwald-Van Slyke pipette, accurately calibrated "to deliver" 0.1 ml.

Pipette with rubber bulb, 0.2 ml.

Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.

Note: The apparatus listed above is the same as that employed in the determination of Carbon Dioxide Capacity (Method C-15) except that the saturation assembly is not required.

Reagents

1. **Double Oxalate Anticoagulant Solution (Heller-Paul).** Dissolve 6 gm crystalline ammonium oxalate ($(NH_4)_2C_2O_4 \cdot H_2O$—Baker & Adamson, A.C.S.) and 4 gm neutral crystalline potassium oxalate ($K_2C_2O_4 \cdot H_2O$—J. T. Baker, CP Analyzed) in distilled water and dilute to 1000 ml.
2. **Mineral Oil** (Marcol GX—Standard Oil Co. of U. S.).
3. **Water, Distilled, Carbon Dioxide-Free.** In a 500 ml flask place 300 ml distilled water; boil for 5 minutes; insert rubber stopper with soda-lime guard and allow to cool to room temperature. This must be used where indicated.
4. **Barium Hydroxide, Ammoniacal (Exton-Schattner-Rose).** In a 100 ml volumetric flask place 2.5 gm crystalline barium hydroxide ($Ba(OH)_2 \cdot 8H_2O$—J. T. Baker, CP Analyzed) and dissolve in 40 ml freshly boiled carbon dioxide-free distilled water (3); add 50 ml ammonium hydroxide (NH_4OH—Baker & Adamson, A.C.S.) and mix; dilute to volume with carbon dioxide-free distilled water (3); filter. Preserve under a 2 cm layer of mineral oil (2) in a resistant-glass bottle. Use only the clear supernatant fluid; do not disturb any white sediment which may form.
5. **Celite** (Johns-Manville Co.).
6. **Hydrochloric Acid, Volumetric 4.5 N.** Add 450 ml concentrated hydrochloric acid (HCl—Baker & Adamson, A.C.S.) to 550 ml carbon dioxide-free distilled water (3) and mix; allow to cool; dilute 20 ml of this approximately 4.5 N solution to 100 ml; titrate against standard 1 N alkali using phenolphthalein as indicator; adjust accordingly and re-titrate—20 ml of a 1:5 dilution of 4.5 N acid require 18 ml of 1 N alkali.
7. **Ammonium Chromate, Ammoniacal (Exton-Schattner-Rose).** In a 100 ml volumetric flask place 250 mg ammonium chromate ($(NH_4)_2CrO_4$—J. T. Baker, CP Analyzed) and dissolve in 40 ml distilled water; add 50 ml ammonium hydroxide

(NH_4OH—Baker & Adamson, A.C.S.) and mix; dilute to volume with distilled water. Preserve in resistant-glass bottle.

8. **Hydrochloric Acid, Concentrated** (HCl—Baker & Adamson, A.C.S.).
9. **Diphenylcarbazide, 1% Alcoholic.** Dissolve 1 gm s-diphenylcarbazide ($(C_6H_5)_2(NH)_4CO$—Eastman #618) in 100 ml 95% ethyl alcohol (C_2H_5OH—U. S. Industrial Chemicals, U.S.P.). Preserve in brown glass bottle away from direct light. The reagent has a pale yellowish color; discard when a red tint develops.

Technic

1. In a 15 ml Pyrex centrifuge tube place 1 ml boiling carbon dioxide-free distilled water (3) by means of a pipette with a rubber bulb—do not aspirate by mouth.
2. Stopper and cool under running tap water.
3. Add 0.1 ml plasma from an Ostwald-Van Slyke pipette and mix thoroughly.
4. Add 0.2 ml ammoniacal barium hydroxide (4) by means of a pipette with a rubber bulb, wiping off any adherent oil.
5. Allow to stand for 5 minutes.
6. Add 10 ml carbon dioxide-free distilled water (3).
7. Add 0.5 mg celite (5) and mix thoroughly by gently inverting the tube two or three times.
8. Centrifuge at low speed (about 800 rpm) for 2 minutes and then at high speed (about 1800 rpm) for 3 minutes—precipitate must be in the tip of the tube—not on the walls.
9. Decant supernatant fluid and drain the tube thoroughly on absorbent paper for 5 minutes—wipe off any liquid around the mouth of the still inverted tube.
10. Add 0.5 ml 4.5 N hydrochloric acid (6) and mix.
11. Place the tube in boiling water for 30 seconds.
12. Cool at once under running tap water.
13. Add 1 ml ammoniacal ammonium chromate (7) and mix.
14. Allow to stand for 5 minutes.
15. Add 10 ml distilled water and repeat Steps 8–9 inclusive.
16. Add 10 ml distilled water and 1 ml concentrated hydrochloric acid (8), stirring with a thin glass rod until the precipitate is completely dissolved; drain the rod into the tube.
17. Transfer the contents of the tube quantitatively to a 100 ml volumetric flask (sample).
18. In another 100 ml volumetric flask (reagent blank) place
    10 ml distilled water
    1 ml concentrated hydrochloric acid (8).
19. To each flask add approximately 75 ml distilled water.
20. Add 0.5 ml 1% diphenylcarbazide (9).
21. Dilute to volume with distilled water and mix.
22. Measure the transmittancy of the sample against the reagent blank set at 100% transmission at a wave length of 480 mμ and obtain the concentration of carbon dioxide from the calibration table for carbon dioxide capacity (Method C-15).

CALIBRATION PROCEDURE

This calibration procedure need not be carried out. The values for carbon dioxide are obtained directly from the calibration table for carbon dioxide capacity (Method C-15).

PROCEDURE FOR USE WITH LINEAR-SCALE PHOTOELECTRIC COLORIMETERS (SHEARD SANFORD TYPE)

TEST PROCEDURE

Exactly as outlined; set the reagent blank at 100 and use a blue filter.

CALIBRATION PROCEDURE

Need not be performed; values obtained directly from calibration table for carbon dioxide capacity.

PROCEDURE FOR USE WITH LOGARITHMIC-SCALE PHOTOELECTRIC COLORIMETERS (KLETT-SUMMERSON TYPE)

TEST PROCEDURE

Exactly as outlined; set the reagent blank at 0 and use a blue filter.

CALIBRATION PROCEDURE

Need not be performed; values obtained directly from calibration table for carbon dioxide capacity.

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CARBON MONOXIDE

A spectrophotometric adaptation based on the methods and modifications of Van Slyke and Robscheit-Robbins (1927), Van Slyke and Hiller (1928), Christman and Randall (1933) and Gettler and Freimuth (1943)

PRINCIPLE

Blood is treated with acid ferricyanide with the resultant liberation of carbon monoxide which is then drawn by aeration through standard palladium chloride. The precipitated metallic palladium is separated and the excess palladium determined by the rose-red color produced upon treatment with potassium iodide, thus indicating the amount of palladium precipitated and indirectly the amount of carbon monoxide in the sample. One mg of palladium chloride is reduced by 0.1577 mg or 0.1261 ml carbon monoxide and since 1 gm of hemoglobin combines with 1.34 ml carbon monoxide each mg of palladium chloride which is reduced indicates the presence of 0.0941 gm of hemoglobin bound with carbon monoxide. Knowing the total hemoglobin concentration, the percentage saturation with carbon monoxide may be readily calculated.

DETERMINATION IN WHOLE BLOOD

SAMPLE

Whole blood—anticoagulant, potassium oxalate. Putrefactive changes of the blood or the presence of embalming fluid cause no interference. The method as outlined is satisfactory for samples representing 5–60% carbon monoxide saturation.

TEST PROCEDURE

Special Apparatus

Apparatus set-up as illustrated (Fig. C-20). All tubes (25 × 150 mm) except "B" are fitted with 2-hole #4 rubber stoppers with inlet and outlet connections. Tube "B" is fitted with a 3-hole rubber stopper through which pass inlet and outlet connections and a small thistle tube with stopcock. The thistle tube is graduated at the 4 ml mark; the stem should extend to just below the outlet tube. Tube "C" contains a 1½ inch layer of perforated glass beads. A Hoffman clamp is placed on the air inlet of tube "A."

Tube "A" removes any carbon monoxide contained in the incoming air.

Tube "B" contains the sample and gas-liberating mixture.

Tube "C" absorbs any hydrogen sulfide which may be admixed with the carbon monoxide; this tube need not be included in the set-up when fresh blood samples are used—only when putrefactive changes may have occurred.

Tube "D" contains the standard palladium chloride.

Tube "E" contains a similar amount of standard palladium chloride; this tube is included to trap any carbon monoxide which may escape from tube "D"—usually the amount of reduced palladium in this tube is negligible; it need not be included when once it is certain that conditions are such that all the gas reacts in tube "D."

Tube "F" is connected to a water pump and is merely a trap for any water which may be sucked back.

Ostwald-Folin pipettes accurately calibrated "to deliver" 2 ml.

Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.

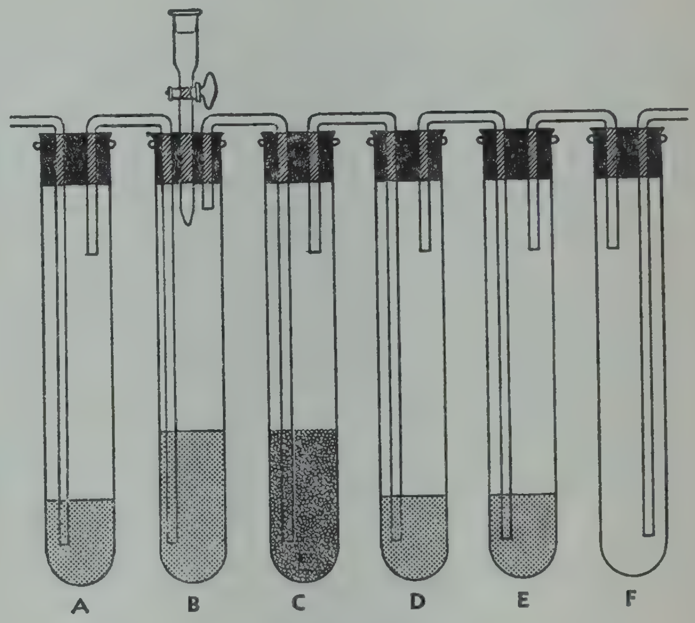

Fig. C-20.

Reagents

1. **Acid Palladium Chloride Solution (Gettler-Freimuth).** Dissolve 0.5 gm palladium chloride ($PdCl_2 \cdot 2H_2O$—Reagent Chemicals, Special) in 0.5 ml concentrated hydrochloric acid (HCl—Baker & Adamson, A.C.S.) and dilute to 50 ml with distilled water.
2. **Caprylic Alcohol** ($C_8H_{17}O_8$—Merck).
3. **Lead Acetate, 10% Aqueous.** Dissolve 10 gm crystalline lead acetate ($Pb(C_2H_3O_2)_2 \cdot 3H_2O$—Baker & Adamson, A.C.S.) in distilled water and dilute to 100 ml.
4. **Palladium Chloride Standard (Christman-Randall).** In a 400 ml beaker place exactly 500 mg palladium chloride ($PdCl_2 \cdot 2H_2O$—Reagent Chemicals, Special); add 150 ml distilled water and 2.5 ml concentrated hydrochloric acid (HCl—Baker & Adamson, A.C.S.); heat until solution is complete; cool; transfer quantitatively to a 500 ml volumetric flask and dilute to volume with distilled water. Preserve in tightly stoppered bottle.

5. **Acid Ferricyanide Reagent (Van Slyke-Hiller).** Add 2 ml 85% lactic acid (a) to 23 ml potassium ferricyanide-saponin solution (b) and mix. This reagent remains stable for several days but is preferably prepared in amounts sufficient for immediate use only.
    (a) Lactic acid, 85% ($HC_3H_5O_3$—Baker & Adamson, Reagent).
    (b) Potassium ferricyanide-saponin solution; dissolve 32 gm crystalline potassium ferricyanide ($K_3Fe(CN)_6$—Baker & Adamson, A.C.S.) in 75 ml distilled water and dilute to 100 ml; add 0.8 gm saponin ($C_{32}H_{54}O_{18}$—Merck) and shake until dissolved.
6. **Aluminum Sulfate, 10% Aqueous.** Dissolve 10 gm crystalline aluminum sulfate ($Al_2(SO_4)_3 \cdot 18H_2O$—Baker & Adamson, Reagent) in distilled water and dilute to 100 ml.
7. **Gum Ghatti, 1% Aqueous.** In a 500 ml cylinder place 500 ml distilled water; suspend 5 gm soluble gum ghatti (Coleman and Bell) in a gauze bag just below the surface of the water; allow to stand for 48 hours; remove gum and adjust volume to 500 ml; mix well and filter. Add a few drops of toluene and preserve in refrigerator.
8. **Potassium Iodide, 15% Aqueous.** Dissolve 7.5 gm crystalline potassium iodide (KI—J. T. Baker, Low in Iodates, Special) in distilled water and dilute to 50 ml. This solution must show no trace of free iodine when tested with starch indicator solution and should be prepared in quantities sufficient for immediate use only.

Technic

Note: With a very low carbon monoxide content a larger sample (up to 5 ml) may be used with no change in the technic other than to increase the amount of acid ferricyanide reagent in Step 6 to 0.75 ml; with a very high content or if only small samples are available use 1 ml blood and 0.15 ml acid ferricyanide reagent; in either case correct the values obtained from the calibration table accordingly.

1. In tube "A" place 5 ml acid palladium chloride solution (1).
2. In tube "B" place
    2 ml oxalated blood from an Ostwald-Folin pipette
    2 drops caprylic alcohol (2).
3. In tube "C", containing a $1^1/_2$ inch layer of perforated glass beads, place 5 ml 10% lead acetate (3).
4. In both tubes "D" and "E" place
    3 ml palladium chloride standard (4)
    2 ml distilled water.
5. Insert stoppers in all tubes and check connections.
6. In the thistle tube place 0.3 ml freshly prepared acid ferricyanide reagent (5) and add distilled water to the 4 ml mark.
7. Allow all the contents except the last drop to drain out of the funnel portion of the thistle tube.
8. Again add distilled water to the 4 ml mark and drain out all except the last drop.
9. Start suction and aerate for 15 minutes very slowly by regulating the Hoffman clamp; agitate tube "B" gently at intervals, the inlet tube always remaining below the level of the blood mixture.
10. Remove tubes "D" and "E" from set-up and stop suction.
11. To both tubes add 0.1 ml 10% aluminum sulfate (6)—mix and allow to stand for 5 minutes; for blood with a high carbon monoxide content an additional 0.1 ml aluminum sulfate in tube "D" may be necessary to produce complete flocculation of the colloidal palladium.
12. Filter contents of both tubes through a 7 cm Whatman #40 paper into a 100 ml volumetric flask (sample), thoroughly rinsing out the tubes with small portions of distilled water and filtering the washings into the flask, keeping the volume in the flask under 25 ml.
13. Remove funnel from flask (but do not discard) and add to the contents of the flask 1 drop caprylic alcohol (2), 2 ml 1% gum ghatti (7) and mix.
14. Add 5 ml freshly prepared 15% potassium iodide (8), while constantly agitating the flask, and mix.
15. Replace funnel in the flask and wash filter paper with 2 ml 15% potassium iodide (8) followed by 2 ml distilled water.
16. Repeat the washing with 2 ml 15% potassium iodide and 2 ml distilled water.
17. Repeat the washing with 1 ml 15% potassium iodide and 2 ml distilled water—remove funnel.
18. Dilute to volume with distilled water.
19. In another 100 ml volumetric flask (standard) place
    3 ml palladium chloride standard (4)
    25 ml distilled water and mix
    1 drop caprylic alcohol (2)
    2 ml 1% gum ghatti (7) and mix
    10 ml 15% potassium iodide (8)
    Dilute to volume with distilled water and mix.
20. Allow the flasks to stand at room temperature for 10 minutes.
21. Measure the transmittancy of the sample against the standard set at *17%* transmission at a wave length of 530 mμ and obtain the milligrams of *precipitated* palladium from the calibration table.

Calculation

To determine carbon monoxide hemoglobin equivalent —
  Multiply the mg of precipitated palladium by 4.705 to obtain carbon monoxide hemoglobin in gm per 100 ml blood

To determine per cent carbon monoxide saturation—
  Determine total hemoglobin by Method H-15 and calculate the carbon monoxide hemoglobin as outlined. Then,

$$\frac{\text{Carbon monoxide hemoglobin}}{\text{Total hemoglobin}} \times 100 = \%\ \text{saturation}$$

CALIBRATION PROCEDURE

Special Apparatus

Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.

Reagents

2. **Caprylic Alcohol.** Same as for Test Procedure.
4. **Palladium Chloride Standard (Christman-Randall).** Same as for Test Procedure.
7. **Gum Ghatti, 1% Aqueous.** Same as for Test Procedure.
8. **Potassium Iodide, 15% Aqueous.** Same as for Test Procedure.
9. **Carbon Monoxide Standards**
    (a) Palladium chloride standard (Christman-Randall). This is the same as Reagent #4.
    (b) Calibration standards. In a series of four accurately calibrated 100 ml volumetric flasks place respectively 0.5, 1, 2 and 3 ml of the palladium chloride standard (9a) and add approximately 50 ml distilled water. These standards contain 0.5, 1, 2 and 3 mg palladium and are respectively equivalent to 2.5, 2, 1 and 0 mg *precipitated* palladium. Prepare just before use and proceed as follows.

Technic

1. To each calibration standard (9b) add in the order named, mixing after each addition
    1 drop caprylic alcohol (2)
    2 ml 1% gum ghatti (7).
2. Add 10 ml freshly prepared 15% potassium iodide (8) slowly and while constantly agitating the flask.
3. Dilute to volume with distilled water and mix.
4. Allow to stand at room temperature for 10 minutes.
5. Set the standard *containing* 3 mg palladium at *17%* transmission at a wave length of 530 mμ and record the transmittancy of the standards *containing* 2, 1 and 0.5 mg palladium. The transmittancy of the 0.5 mg standard should be approximately 67%.
6. Plot the observed values on semi-log graph paper—the points fall on a straight line which intersects the transmission axis at 17% and slopes upward (Calibration Curve C-20).
7. Substitute the *precipitated* palladium values 2.5, 2, 1 and 0 mg (the amount actually reduced) for the values of the standards *containing* 0.5, 1, 2 and 3 mg palladium and prepare a calibration table from these T-C values obtained from the plotted curve.

PROCEDURE FOR USE WITH LINEAR-SCALE PHOTOELECTRIC COLORIMETERS (SHEARD-SANFORD TYPE)

TEST PROCEDURE

Exactly as outlined; set the standard at 20 and use a green filter.

CALIBRATION PROCEDURE

Exactly as outlined—use a green filter; plot the observed readings on semi-log graph paper—the points fall on a straight line which intersects the transmission axis at 20% and slopes upward.

PROCEDURE FOR USE WITH LOGARITHMIC-SCALE PHOTOELECTRIC COLORIMETERS (KLETT-SUMMERSON TYPE)

TEST PROCEDURE

Exactly as outlined; set the standard at 300 and use a green filter.

CALIBRATION PROCEDURE

Exactly as outlined—use a green filter; plot the observed readings on linear graph paper—the points fall on a straight line which intersects the instrument reading axis at 300 and slopes downward.

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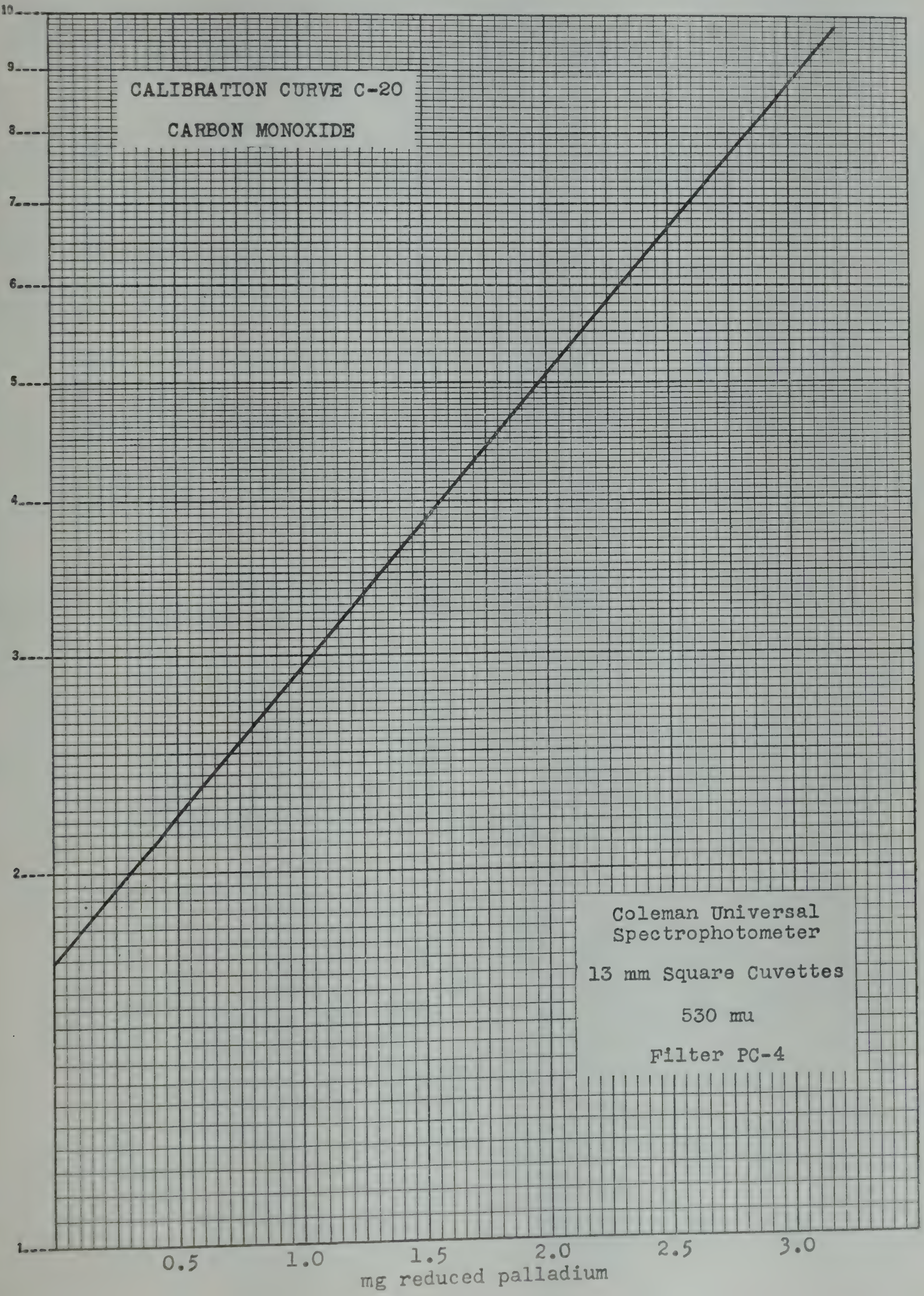
NOTES


CALIBRATION CURVE C-20
CARBON MONOXIDE
Coleman Universal Spectrophotometer
13 mm Square Cuvettes
530 mu
Filter PC-4
10
9
8
7
6
5
4
3
2
1
0.5
1.0
1.5
2.0
2.5
3.0
mg reduced palladium

Meter Reading	0	1	2	3	4	5	6	7	8	9
0										
10										
20										
30										
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50										
60										
70										
80										
90										

CHLORIDE

A spectrophotometric adaptation based on the methods and modifications of Sendroy (1937; 1939), Hoffman (1941), Van Slyke and Hiller (1947) and Stiff (1948)

PRINCIPLE

Plasma or serum is diluted with phosphoric-tungstic acid and shaken with silver iodate. The proteins are precipitated and the chloride removed as solid silver chloride. By treating the equivalent iodate remaining in solution with sodium iodide, six iodine equivalents are liberated for each equivalent of original chloride present. The yellow color of the free iodine is measured directly.

DETERMINATION IN PLASMA OR SERUM

SAMPLE

Plasma or serum from blood withdrawn during postabsorptive state. There must be no trace of hemolysis and the red cells must be separated as soon as possible. For plasma analyses heparin or potassium oxalate must be used as an anticoagulant.

TEST PROCEDURE

Special Apparatus

Centrifuge tubes, 15 ml, glass-stoppered.
Van Slyke-Neill micro pipettes accurately calibrated "to deliver" 0.2 ml.
Glass measuring spoons (the type described by Van Slyke, Hiller, Weisiger and Cruz is preferable).
Ostwald-Folin pipettes accurately calibrated "to deliver" 0.5 ml.
Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.

Note: All glassware must be thoroughly cleaned with dichromate-sulfuric acid solution (see Appendix), rinsed with distilled water and oven dried before use.

Reagents

Note: All reagents must be chloride-free or the chloride content must be known.

1. **Phosphoric-Tungstic Acid Solution (Van Slyke-Hiller).** In a 1000 ml volumetric flask place 6 gm low-chloride sodium tungstate ($Na_2WO_4 \cdot 2H_2O$—Mallinckrodt, Folin Special); add about 800 ml 0.15 M phosphoric acid (a) and shake until dissolved; dilute to volume with same.
   (a) Phosphoric acid, 0.15 M; dilute 10 ml 85% chloride-free phosphoric acid (H_3PO_4—Baker & Adamson, A.C.S.) to 1000 ml with distilled water.
2. **Silver Iodate** ($AgIO_3$—Merck, Special, Suitable for Micro Analysis).
3. **Sodium Iodide, 20% Aqueous.** Dissolve 1 gm low-chloride sodium iodide (NaI—J. T. Baker, CP Analyzed) in 5 ml distilled water. Prepare the amount required just before use and discard any excess.

Note: The use of sodium iodide in previously weighed capsules or vials is not recommended—the possibility of free iodine formation is enhanced. Furthermore, sodium iodide is extremely hygroscopic and may adhere to the sides of the container. Every lot of sodium iodide must be tested frequently with starch indicator solution for the presence of free iodine; it should be preserved in a brown glass bottle away from direct light.

Technic

Note: For concentrations of chloride less than 30 meq per liter, use 0.4 ml sample and 4.6 ml phosphoric-tungstic acid solution (1) in Step 1 and divide the values obtained from the calibration table by 2; for concentrations in excess of 150 meq per liter, use 0.1 ml sample and 4.9 ml phosphoric-tungstic acid solution (1) in Step 1 and multiply the values obtained from the calibration table by 2.

1. In a 15 ml glass-stoppered centrifuge tube (sample) place
   4.8 ml phosphoric-tungstic acid solution (1)
   0.2 ml sample from a Van Slyke-Neill pipette, added drop by drop, while constantly agitating tube.
2. In another 15 ml glass-stoppered centrifuge tube (reagent blank) place
   5 ml phosphoric-tungstic acid solution (1).
3. To each tube add approximately 60 mg silver iodate (2) from a small glass measuring spoon.
4. Stopper the tubes and shake vigorously for 1 minute.
5. Centrifuge at 2500 rpm for 1 minute.
6. Transfer 0.5 ml of the clear supernatant centrifugates to correspondingly marked 50 ml volumetric flasks.
7. Add about 4 ml distilled water and mix.
8. Add 1 ml freshly prepared 20% sodium iodide (3) and mix.
9. Dilute to volume with distilled water and mix.
10. Place in water bath at 25° C. for 10 minutes.
11. Measure the transmittancy of the sample against the reagent blank set at 100% transmission at a wave length of 420 mμ and obtain the concentration of chloride expressed as sodium chloride from the calibration table.

CALIBRATION PROCEDURE.

Special Apparatus

As listed under the Test Procedure.

Reagents

1. **Phosphoric-Tungstic Acid Solution (Van Slyke-Hiller).** Same as for Test Procedure.
2. **Silver Iodate.** Same as for Test Procedure.

3. **Sodium Iodide, 20% Aqueous.** Same as for Test Procedure.
4. **Chloride Standards**
    (a) Sodium chloride stock standard (0.1 M). In a 100 ml volumetric flask place 584.5 mg sodium chloride (NaCl—Merck, Special for Biological Work); add about 75 ml distilled water and shake until dissolved; dilute to volume.
    (b) Dilute standard (0.006 M). In a 100 ml volumetric flask place 6 ml stock standard (4a) and dilute to volume with phosphoric-tungstic acid solution (1). Prepare just before use.
    (c) Calibration standards. In a series of six accurately calibrated 10 ml volumetric flasks place respectively 0, 2, 4, 6, 8 and 10 ml of the dilute standard (4b) and dilute to volume with phosphoric-tungstic acid solution (1). These standards represent concentrations of sodium chloride equivalent to 0, 30, 60, 90, 120 and 150 meq per liter. Prepare just before use.

Technic

1. Transfer 5 ml of each calibration standard (4c) to a correspondingly marked 15 ml glass-stoppered centrifuge tube.
2. Proceed exactly as outlined in the Test Procedure, Steps 3–10 inclusive.
3. Set the zero concentration standard at 100% transmission at a wave length of **420** mμ and record the transmittancy of the 30, 60, 90, 120 and 150 meq per liter standards. The transmittancy of the 150 meq standard should be approximately 24%.
4. Plot the observed values on semi-log graph paper —the points fall on a slight curve which passes through the origin (Calibration Curve C-25).
5. Make an enlargement of the above calibration curve by spreading the number of concentration divisions between the 90 and 120 meq per liter concentrations (Calibration Curve C-25A). This enables a more accurate determination of the values within the normal range.
6. Prepare a calibration table from the T-C values obtained from the plotted curves.

CONVERSION FACTOR

To convert sodium chloride in milliequivalents per liter to sodium chloride in milligrams per 100 ml multiply by 5.8454.

Method C-25b

DETERMINATION IN URINE

SAMPLE

Aliquot of a 24-hour specimen. Keep cold during collection.

TEST PROCEDURE

Special Apparatus

As listed under the Test Procedure for blood.

Reagents

1. **Phosphoric-Tungstic Acid Solution (Van Slyke-Hiller).** Same as for blood.
2. **Silver Iodate.** Same as for blood.
3. **Sodium Iodide, 20% Aqueous.** Same as for blood.

Technic

Note: For concentrations of chloride less than 30 meq per liter, use 0.4 ml urine and 4.6 ml phosphoric-tungstic acid solution (1) and divide the values obtained from the calibration table for blood by 2; for very low concentrations even larger samples may be necessary; for concentrations in excess of 150 meq per liter, dilute 5 ml filtered urine to 10 ml with distilled water before proceeding as outlined and multiply the values obtained from the calibration table by 2.

1. Filter a small portion of thoroughly mixed urine.
2. Treat 0.2 ml filtered urine exactly as outlined in the Test Procedure for blood, Steps 1–10 inclusive.
3. Measure the transmittancy of the sample against the reagent blank set at 100% transmission at a wave length of **420** mμ and obtain the concentration of chloride expressed as sodium chloride directly from the calibration table for blood.

Calculation

$$\frac{\text{Volume of 24-hour specimen}}{100} \times \text{Sodium chloride in mg per 100 ml} = \text{Mg sodium chloride excreted in 24 hours}$$

Method C-25c

DETERMINATION IN CEREBROSPINAL FLUID

SAMPLE

The admixture of whole or hemolyzed blood or the presence of partially decomposed blood vitiates the results.

TEST PROCEDURE

Special Apparatus

Van Slyke-Neill micro pipettes accurately calibrated "to deliver" 0.1 ml.

Other apparatus the same as listed under the Test Procedure for blood.

Reagents

1. **Phosphoric-Tungstic Acid Solution (Van Slyke-Hiller).** Same as for blood.
2. **Silver Iodate.** Same as for blood.
3. **Sodium Iodide, 20% Aqueous.** Same as for blood.

Technic

Note: For concentrations of chloride less than 70 meq per liter, follow the procedure exactly as outlined for blood and obtain the values directly from the calibration table for blood; for normal and higher concentrations, use the following procedure.

1. In a 15 ml glass-stoppered centrifuge tube (sample) place
   4.9 ml phosphoric-tungstic acid solution (1)
   0.1 ml cerebrospinal fluid from a Van Slyke-Neill pipette, added drop by drop, while constantly agitating the tube.
2. Proceed exactly as outlined in the Test Procedure for blood, Steps 2–10 inclusive.
3. Measure the transmittancy of the sample against the reagent blank set at 100% transmission at a wave length of 420 $m\mu$ and multiply the value from the calibration table for blood by 2 to obtain the concentration of chloride expressed as sodium chloride in meq per liter.

Method C-25d

DETERMINATION IN GASTRIC CONTENTS

SAMPLE

Aliquot of specimen withdrawn by any procedure not utilizing sodium chloride or hydrochloric acid.

TEST PROCEDURE

Special Apparatus

As listed under the Test Procedure for blood.

Reagents

1. **Phosphoric-Tungstic Acid Solution (Van Slyke-Hiller).** Same as for blood.
2. **Silver Iodate.** Same as for blood.
3. **Sodium Iodide, 20% Aqueous.** Same as for blood.

Technic

Note: For concentrations of chloride less than 30 meq per liter, use 0.4 ml gastric contents and 4.6 ml phosphoric-tungstic acid solution (1) and divide the values obtained from the calibration table for blood by 2; for very low concentrations even larger samples may be necessary; for concentrations in excess of 150 meq per liter, use 0.1 ml sample and 4.9 ml phosphoric-tungstic acid solution (1) in Step 2 and multiply the values obtained from the calibration table by 2.

1. Centrifuge a portion of thoroughly mixed gastric contents.
2. In a 15 ml glass-stoppered centrifuge tube (sample) place
   4.8 ml phosphoric-tungstic acid solution (1)
   0.2 ml clear supernatant centrifugate from a Van Slyke-Neill pipette, added drop by drop, while constantly agitating the tube.
3. Proceed exactly as outlined in the Test Procedure for blood, Steps 2–10 inclusive.
4. Measure the transmittancy of the sample against the reagent blank set at 100% transmission at a wave length of 420 $m\mu$ and obtain the concentration of chloride expressed as sodium chloride directly from the calibration table for blood.

PROCEDURE FOR USE WITH LINEAR-SCALE PHOTOELECTRIC COLORIMETERS (SHEARD-SANFORD TYPE)

TEST PROCEDURE

Exactly as outlined; set the reagent blank at 100 and use a blue filter.

CALIBRATION PROCEDURE

Exactly as outlined—use a blue filter; plot the observed readings on semi-log graph paper—the points fall on a very slight curve passing through the origin.

PROCEDURE FOR USE WITH LOGARITHMIC-SCALE PHOTOELECTRIC COLORIMETERS (KLETT-SUMMERSON TYPE)

TEST PROCEDURE

Exactly as outlined; set the reagent blank at 0 and use a blue filter.

CALIBRATION PROCEDURE

Exactly as outlined—use a blue filter; plot the observed readings on linear graph paper—the points fall on a very slight curve passing through the origin.

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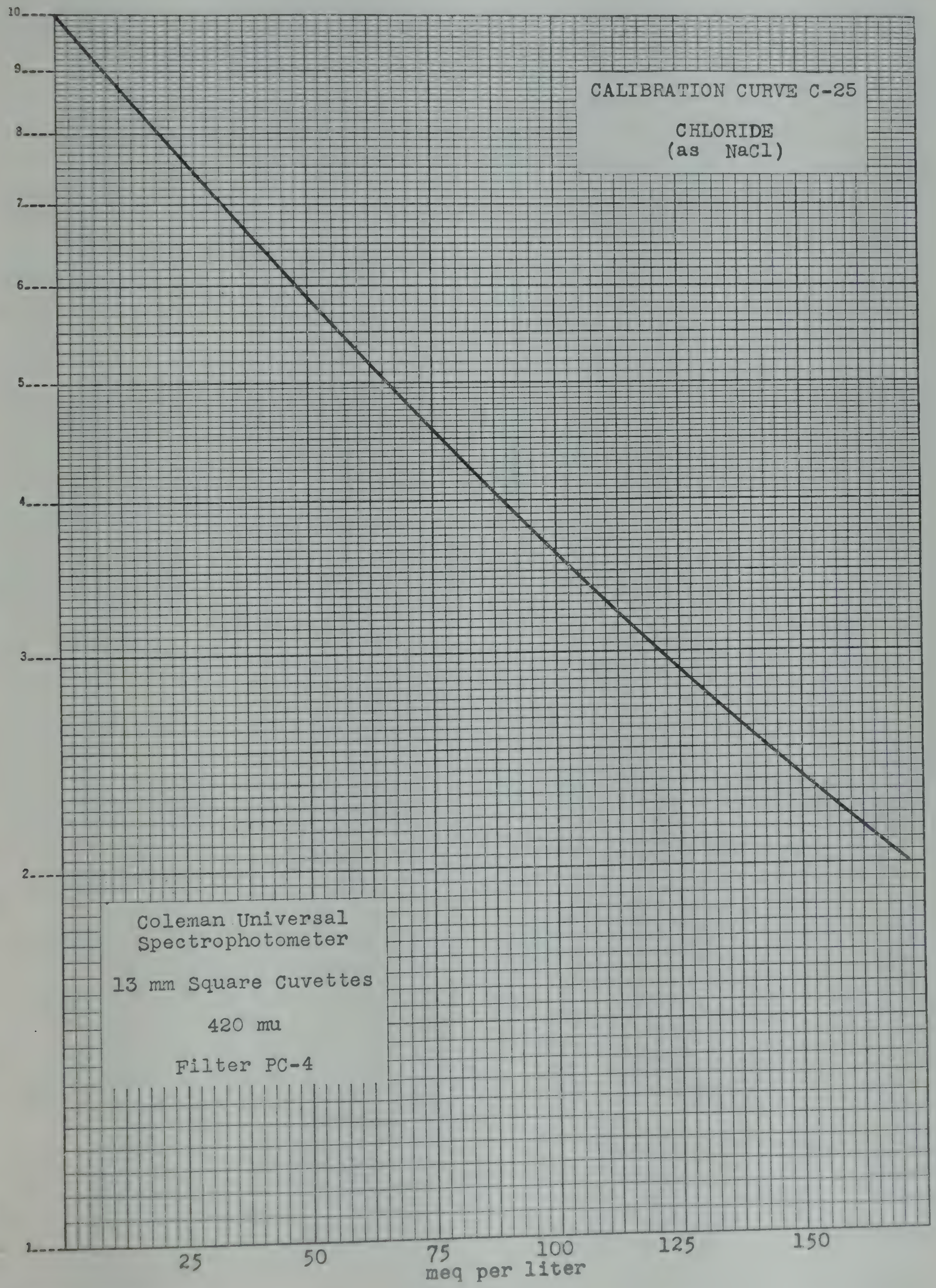
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420 mu
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9
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5
4
3
2
1
25
50
75
100
125
150
meq per liter

Meter Reading	0	1	2	3	4	5	6	7	8	9
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50										
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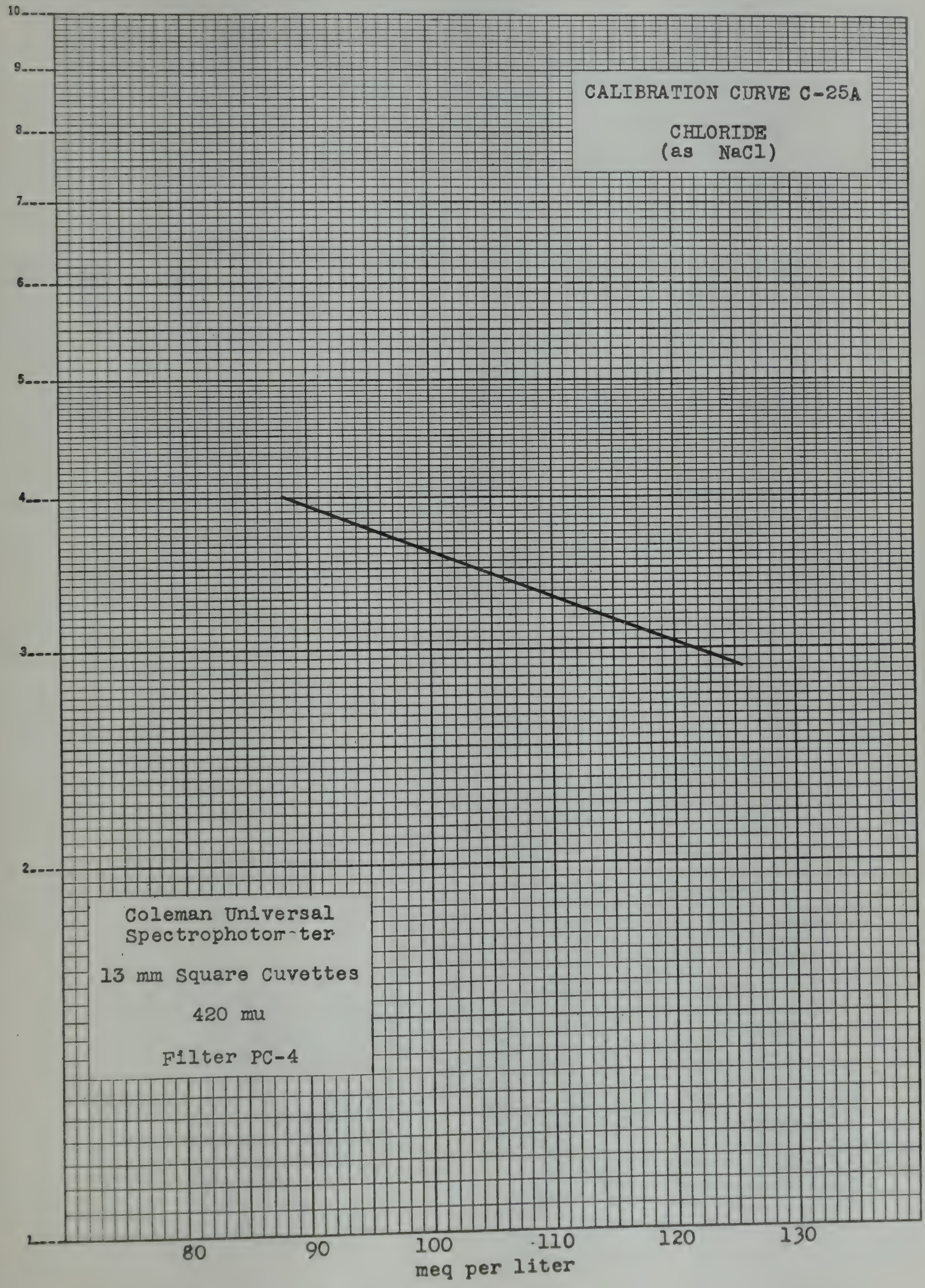
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3
2
1
80
90
100
110
120
130
meq per liter

Meter Reading	0	1	2	3	4	5	6	7	8	9
0										
10										
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90										

CHOLESTEROL, TOTAL

A spectrophotometric adaptation based on the methods and modifications of Bloor (1916; 1928), Bloor, Pelkan and Allen (1922), Sackett (1925), Schube (1932), Schoenheimer and Sperry (1934), Sperry (1938) and Sobel and Mayer (1945)

PRINCIPLE

Total cholesterol is extracted and proteins are precipitated simultaneously by means of an alcohol:ether mixture. After evaporation of the solvent, the residue is taken up with chloroform and treated with acetic anhydride and sulfuric acid (Liebermann-Burchard reaction). The following procedure employs the very stable final yellow color developed at 50° C.

DETERMINATION IN PLASMA OR SERUM

SAMPLE

Serum or heparinized plasma from blood withdrawn during post-absorptive state—analyses preferably made on plasma. Whole blood and oxalated plasma should not be used. The plasma or serum must be separated from the red cells as soon as possible. The determination of total cholesterol may be made any time within 24 hours after withdrawal of the blood.

TEST PROCEDURE

Special Apparatus

Fister cholesterol tubes—15 ml; glass-stoppered; the 5, 7, 10 and 12 ml graduations must be accurately calibrated.

Ostwald-Van Slyke micro pipettes accurately calibrated "to deliver" 0.2 ml.

Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.

Note: All apparatus should be thoroughly oven-dried before use. If pipettes are to be used for measuring chloroform solutions, they must be accurately calibrated with chloroform and not with distilled water.

Reagents

Note: All reagents must be anhydrous.

1. **Alcohol:Ether Mixture, 3:1 (Bloor).** Mix 3 volumes absolute ethyl alcohol (C_2H_5OH—U.S. Industrial Chemicals, U.S.P.) with 1 volume anhydrous peroxide-free ether ($(C_2H_5)_2O$—Mallinckrodt, AR).

   Note: All samples of ether should be checked for the presence of peroxide as follows: Shake a small sample of ether with one-half volume freshly prepared 10% potassium iodide slightly acidified with concentrated sulfuric acid; if more than a very faint yellow color develops, the ether must be purified by washing with 10% sodium sulfite followed by several washings with distilled water, and distilled over calcium chloride. Preserve in the dark in a tightly stoppered brown glass bottle containing a coil of pure iron wire.

2. **Chloroform, Anhydrous** ($CHCl_3$—Baker & Adamson, A.C.S.).

   Note: Chloroform may be tested for decomposition products by overlaying 1 ml with 1 ml baryta water—no film should form at the interface. Decomposed chloroform may be re-distilled and 0.5% ethyl alcohol added to the final distillate as a preservative.

3. **Acetic Anhydride-Sulfuric Acid Reagent, 10:1 (Bloor).** In a 10 ml mixing cylinder place 10 ml acetic anhydride (a), slowly add 1 ml concentrated sulfuric acid (b) and mix by repeated inversion; place the cylinder in a beaker of cold water and cool to room temperature. Use within 10 minutes after preparation.
   - (a) Acetic anhydride ($(CH_3CO)_2O$—Baker & Adamson, A.C.S.); if not absolutely colorless or if a slight sediment is present or if the chromogenic power has deteriorated due to long standing, the anhydride must be re-distilled.
   - (b) Sulfuric acid, concentrated (H_2SO_4—Grasselli, A.C.S.); the acid must be absolutely colorless; it should be preserved in small quantities in bottles with tightly fitting glass stoppers; slightly more than the amount required for immediate use should be decanted into a clean dry beaker and any excess discarded.

Technic

Note: For concentrations of total cholesterol in excess of 350 mg per 100 ml, use 0.1 ml sample in Step 1 and multiply the values obtained from the calibration table by 2. It is essential that moisture be excluded throughout the procedure.

1. In a special dry glass-stoppered cholesterol tube place
   12 ml alcohol:ether mixture (1)
   0.2 ml sample from an Ostwald-Van Slyke micro pipette, added drop by drop while constantly agitating the tube.
2. Stopper the tube tightly and shake vigorously for 1 minute.
3. Place the stoppered tube in a horizontal position and allow to remain in this position for 30 minutes.
4. Centrifuge at 2000 rpm for 5 minutes.
5. Decant the clear supernatant extract into a 30 ml beaker, draining off the last drop.
6. Place the beaker on a sand or water bath and evaporate to *just* dryness; heating must not be prolonged beyond the point where the last trace of moisture disappears nor must any odor of acrolein be discernible.

Note: If evaporation is carried out on a water bath, care must be taken to prevent any moisture from entering the beaker.

7. Immediately after removing the beaker from the bath and while still hot, extract with repeated small portions of chloroform (2), placing all the extracts in a special dry glass-stoppered cholesterol tube (sample), keeping the volume in the tube under 10 ml.
8. Allow to cool and dilute to 10 ml with chloroform (2).
9. In another special dry glass-stoppered cholesterol tube (reagent blank) place
   10 ml chloroform (2).
10. To each tube add 2 ml freshly prepared and cooled acetic anhydride-sulfuric acid reagent (3), placing the tip of the pipette just above the surface of the chloroform and allowing the reagent to flow down the side of the tube; mix.
11. Stopper the tubes tightly and place in water bath at 50° C. for 45 minutes—the level of the water in the bath should not be above the shoulders of the tubes.
12. Without removing the stoppers, place the tubes in a beaker of cold water and cool to room temperature.
13. Wipe all the water off the outside of the tubes and transfer to correspondingly marked cuvettes.
14. Measure the transmittancy of the sample against the reagent blank set at 100% transmission at a wave length of 415 mμ and obtain the concentration of total cholesterol from the calibration table.

CALIBRATION PROCEDURE

Special Apparatus

Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.
Fister cholesterol tubes—15 ml; glass-stoppered; the 5, 7, 10 and 12 ml graduations must be accurately calibrated.
Burettes with capillary tips—Normax; 10 ml in 0.1 ml.

Reagents

2. **Chloroform, Anhydrous.** Same as for Test Procedure.
3. **Acetic Anhydride-Sulfuric Acid Reagent, 10:1 (Bloor).** Same as for Test Procedure.
4. **Total Cholesterol Standards**

Note: When preparing any standard with chloroform as a solvent, note the temperature and always bring the standard to this temperature before use. In the author's laboratory a temperature of 21° C. is always maintained when working with chloroform solutions.

(a) Cholesterol stock standard (1 ml contains 1 mg cholesterol). In a 100 ml volumetric flask place 100 mg recrystallized free cholesterol ($C_{27}H_{45}OH$—Reagent Chemicals, Special); dissolve in chloroform (2) and dilute to volume with same. Preserve in a glass-stoppered bottle.

(b) Dilute standard (1 ml contains 0.08 mg cholesterol). In a 50 ml volumetric flask place 4 ml stock standard (4a) and dilute to volume with chloroform (2). Prepare day of use and keep in a glass-stoppered bottle.

(c) Calibration standards. In a series of eight accurately calibrated special dry glass-stoppered cholesterol tubes place respectively 0, 1, 2, 3, 4, 5, 8 and 10 ml of the dilute standard (4b) measured from a 10 ml burette; add 10, 9, 8, 7, 6, 5, 2 and 0 ml chloroform (2) respectively, measured from another 10 ml burette, bringing the volume in each tube to 10 ml. These standards represent concentrations of total cholesterol equivalent to 0, 40, 80, 120, 160, 200, 320 and 400 mg per 100 ml sample. Prepare just before use.

Technic

1. Treat each calibration standard (4c) exactly as outlined in the Test Procedure, Steps 10–13 inclusive.
2. Set the zero concentration standard at 100% transmission at a wave length of 415 mμ and record the transmittancy of the 40, 80, 120, 160, 200, 320 and 400 mg per cent standards. The transmittancy of the 400 mg standard should be approximately 18%.
3. Plot the observed values on semi-log graph paper—the points fall on a straight line which passes through the origin (Calibration Curve C-30).
4. Prepare a calibration table from the T-C values obtained from the plotted curve.

PROCEDURE FOR USE WITH LINEAR-SCALE PHOTOELECTRIC COLORIMETERS (SHEARD-SANFORD TYPE)

TEST PROCEDURE

Exactly as outlined; set the reagent blank at 100 and use a blue filter.

CALIBRATION PROCEDURE

Exactly as outlined—use a blue filter; plot the observed readings on semi-log graph paper—the points fall on a straight line passing through the origin.

PROCEDURE FOR USE WITH LOGARITHMIC-SCALE PHOTOELECTRIC COLORIMETERS (KLETT-SUMMERSON TYPE)

TEST PROCEDURE

Exactly as outlined; set the reagent blank at 0 and use a blue filter.

CALIBRATION PROCEDURE

Exactly as outlined—use a blue filter; plot the observed readings on linear graph paper—the points fall on a straight line passing through the origin.

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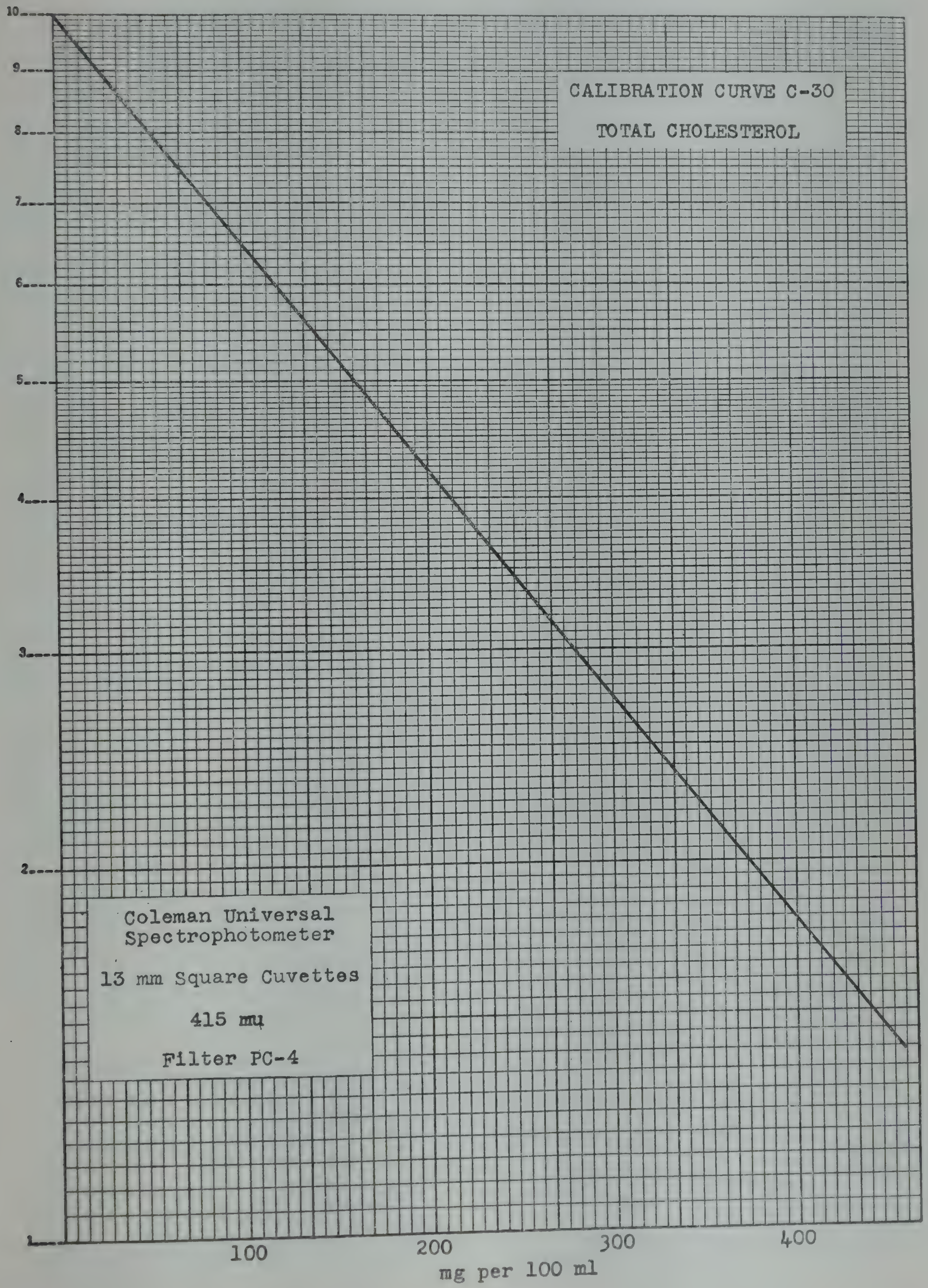
CALIBRATION CURVE C-30
TOTAL CHOLESTEROL
Coleman Universal Spectrophotometer
13 mm Square Cuvettes
415 mμ
Filter PC-4
10
9
8
7
6
5
4
3
2
1
100
200
300
400
mg per 100 ml

Meter Reading	0	1	2	3	4	5	6	7	8	9
0										
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CHOLESTEROL, ESTER AND FREE

A spectrophotometric adaptation based on the methods and modifications of Bloor (1916; 1928), Bloor and Knudson (1916), Bloor, Pelkan and Allen (1922), Sackett (1925), Schube (1932), Schoenheimer and Sperry (1934), Sperry (1938) and Sobel and Mayer (1945)

PRINCIPLE

Total cholesterol is extracted and proteins are precipitated simultaneously by means of an alcohol: ether mixture. The free cholesterol is precipitated by digitonin and after evaporation of the solvent, the cholesterol ester fraction is extracted with hot petroleum ether. The ether extract is evaporated and the residue taken up with chloroform. The analysis is then carried out as outlined in the method for total cholesterol (Method C-30), the same analytical principles being involved. Free cholesterol is the difference between the total cholesterol and the ester cholesterol.

DETERMINATION IN PLASMA OR SERUM

SAMPLE

Serum or heparinized plasma from blood withdrawn during post-absorptive state—analyses preferably made on plasma. Whole blood and oxalated plasma should not be used. The plasma or serum must be separated from the red cells as soon as possible. The determination of the ester fraction must be started within 30 minutes after withdrawal of the blood and carried through the digitonin precipitation without delay.

TEST PROCEDURE

Special Apparatus

Fister cholesterol tubes—15 ml; glass-stoppered; the 5, 7, 10 and 12 ml graduations must be accurately calibrated.

Ostwald-Van Slyke micro pipettes accurately calibrated "to deliver" 0.2 ml.

Cotton, non-absorbent, petroleum ether-washed.

Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.

Note: All apparatus should be thoroughly oven-dried before use. If pipettes are to be used for measuring chloroform solutions, they must be accurately calibrated with chloroform and not with distilled water.

Reagents

1. **Alcohol: Ether Mixture, 3:1 (Bloor).** Mix 3 volumes absolute ethyl alcohol (C_2H_5OH—U.S. Industrial Chemicals, U.S.P.) with 1 volume anhydrous peroxide-free ether ($(C_2H_5)_2O$—Mallinckrodt, AR).

Note: All samples of ether should be checked for the presence of peroxide as follows: Shake a small sample of ether with one-half volume freshly prepared 10% potassium iodide slightly acidified with concentrated sulfuric acid; if more than a very faint yellow color develops, the ether must be purified by washing with 10% sodium sulfite followed by several washings with distilled water, and distilled over calcium chloride. Preserve in the dark in a tightly stoppered brown glass bottle containing a coil of pure iron wire.

2. **Digitonin, 1% Alcoholic.** Dissolve 1 gm recrystallized digitonin ($C_{54}H_{92}O_{28}$—Reagent Chemicals, Special) in 100 ml 50% ethyl alcohol (a) at 60° C.; allow to stand for 24 hours and filter off any precipitate that may have formed. The filtrate should be crystal clear and remains stable for at least six months.
   (a) Ethyl alcohol, 50% aqueous; in a 100 ml mixing cylinder place 55 ml 95% ethyl alcohol (C_2H_5OH—U.S. Industrial Chemicals, U.S.P.) and dilute to volume with distilled water; allow to cool and adjust volume.
3. **Petroleum Ether** (B.R. 30°–60°—Baker & Adamson, A.C.S.).
4. **Chloroform, Anhydrous** ($CHCl_3$—Baker & Adamson, A.C.S.).

Note: Chloroform may be tested for decomposition products by overlaying 1 ml with 1 ml baryta water—no film should form at the interface. Decomposed chloroform may be redistilled and 0.5% ethyl alcohol added to the final distillate as a preservative.

5. **Acetic Anhydride-Sulfuric Acid Reagent, 10:1 (Bloor).** In a 10 ml mixing cylinder place 10 ml acetic anhydride (a), slowly add 1 ml concentrated sulfuric acid (b) and mix by repeated inversion; place the cylinder in a beaker of cold water and cool to room temperature. Use within 10 minutes after preparation.
   (a) Acetic anhydride ($(CH_3CO)_2O$—Baker & Adamson, A.C.S.); if not absolutely colorless or if a slight sediment is present or if the chromogenic power has deteriorated due to long standing, the anhydride must be re-distilled.
   (b) Sulfuric acid, concentrated (H_2SO_4—Grasselli, A.C.S.); the acid must be absolutely colorless; it should be preserved in small quantities in bottles with tightly fitting glass stoppers; slightly more than the amount required for immediate use should be decanted into a clean dry beaker and any excess discarded.

Technic

Note: It is essential that moisture be excluded throughout the procedure.

1. In a special dry glass-stoppered cholesterol tube place
   12 ml alcohol: ether mixture (1)
   0.2 ml sample from an Ostwald-Van Slyke micro pipette, added drop by drop while constantly agitating the tube.

2. Stopper the tube tightly and shake vigorously for 1 minute.
3. Place the stoppered tube in a horizontal position and allow to remain in this position for 30 minutes.
4. Centrifuge at 2000 rpm for 5 minutes.
5. Decant the clear supernatant extract into a 30 ml beaker, draining off the last drop.
6. To the alcohol: ether extract in the beaker add 1 ml 1% digitonin (2), mix thoroughly and allow to stand in the incubator at 37° C. for 2 hours—longer does no harm.
7. Place the beaker on a sand or water bath and evaporate to dryness, allowing the beaker to remain on the bath for *one* minute after the last trace of moisture has disappeared.

Note: If evaporation is carried out on a water bath, care must be taken to prevent any moisture from entering the beaker.

8. Remove the beaker from the bath, add 10 ml petroleum ether (3) and at once replace on bath.
9. Evaporate to half-volume and filter the extract through a small piece of ether-washed non-absorbent cotton into another 30 ml beaker.
10. On the residue in the original beaker repeat Steps 8 and 9 twice more, each time adding the extract to the second beaker.
11. Evaporate the combined filtrates to *just* dryness.
12. Immediately after removing the beaker from the bath and while still hot, extract with repeated small portions of chloroform (4), placing all the extracts in a special dry glass-stoppered cholesterol tube (sample), keeping the volume in the tube under 5 ml.
13. Allow to cool and dilute to 5 ml with chloroform (4).
14. In another special dry glass-stoppered cholesterol tube (reagent blank) place
    5 ml chloroform (4).
15. To each tube add 2 ml freshly prepared and cooled acetic anhydride-sulfuric acid reagent (5), placing the tip of the pipette just above the surface of the chloroform and allowing the reagent to flow down the side of the tube; mix.
16. Stopper the tubes tightly and place in water bath at 50° C. for 45 minutes—the level of the water in the bath should not be above the shoulders of the tubes.
17. Without removing the stoppers, place the tubes in a beaker of cold water and cool to room temperature.
18. Wipe all the water off the outside of the tubes and transfer to correspondingly marked cuvettes.
19. Measure the transmittancy of the sample against the reagent blank set at 100% transmission at a wave length of 415 mμ and obtain the concentration of ester cholesterol from the calibration table.

Calculation

Total cholesterol in mg per 100 ml − Ester cholesterol in mg per 100 ml = Free cholesterol in mg per 100 ml

Note: The total cholesterol concentration is determined as outlined in Method C-30.

CALIBRATION PROCEDURE

Special Apparatus

Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.

Fister cholesterol tubes—15 ml; glass-stoppered; the 5, 7, 10 and 12 ml graduations must be accurately calibrated.

Burettes with capillary tips—Normax; 10 ml in 0.1 ml.

Reagents

4. **Chloroform, Anhydrous.** Same as for Test Procedure.
5. **Acetic Anhydride-Sulfuric Acid Reagent, 10:1 (Bloor).** Same as for Test Procedure.
6. **Cholesterol Ester Standards**

Note: When preparing any standard with chloroform as a solvent, note the temperature and always bring the standard to this temperature before use. In the author's laboratory a temperature of 21° C. is always maintained when working with chloroform solutions.

(a) Cholesterol ester stock standard (1 ml equivalent to 1 mg cholesterol). In a 100 ml volumetric flask place 110.8 mg cholesteryl acetate ($CH_3COOC_{27}H_{45}$—Reagent Chemicals, Special) or 161.7 mg cholesteryl palmitate ($CH_3(CH_2)_{14}COOC_{27}H_{45}$—Reagent Chemicals, Special); dissolve in chloroform (4) and dilute to volume with same. Preserve in a glass-stoppered bottle.

(b) Dilute standard (1 ml equivalent to 0.08 mg cholesterol). In a 50 ml volumetric flask place 4 ml stock standard (6a) and dilute to volume with chloroform (4). Prepare day of use and keep in a glass-stoppered bottle.

(c) Calibration standards. In a series of eight accurately calibrated 10 ml volumetric flasks place respectively 0, 1, 2, 3, 4, 5, 8 and 10 ml of the dilute standard (6b) measured from a 10 ml burette; dilute to volume with chloroform (4). These standards represent concentrations of ester cholesterol equivalent to 0, 20, 40, 60, 80, 100, 160 and 200 mg per 100 ml sample. Prepare just before use.

Technic

1. Transfer 5 ml of each calibration standard (6c) to a correspondingly marked special dry glass-stoppered cholesterol tube.
2. Proceed exactly as outlined in the Test Procedure, Steps 15–18 inclusive.
3. Set the zero concentration standard at 100% transmission at a wave length of **415** mμ and record the transmittancy of the 20, 40, 60, 80, 100, 160 and 200 mg per cent standards. The transmittancy of the 200 mg standard should be approximately 23%.
4. Plot the observed values on semi-log graph paper—the points fall on a straight line which passes through the origin (Calibration Curve C-31).
5. Prepare a calibration table from the T-C values obtained from the plotted curve.

PROCEDURE FOR USE WITH LINEAR-SCALE PHOTOELECTRIC COLORIMETERS (SHEARD-SANFORD TYPE)

TEST PROCEDURE

Exactly as outlined; set the reagent blank at 100 and use a blue filter.

CALIBRATION PROCEDURE

Exactly as outlined—use a blue filter; plot the observed readings on semi-log graph paper—the points fall on a straight line passing through the origin.

PROCEDURE FOR USE WITH LOGARITHMIC-SCALE PHOTOELECTRIC COLORIMETERS (KLETT-SUMMERSON TYPE)

TEST PROCEDURE

Exactly as outlined; set the reagent blank at 0 and use a blue filter.

CALIBRATION PROCEDURE

Exactly as outlined—use a blue filter; plot the observed readings on linear graph paper—the points fall on a straight line passing through the origin.

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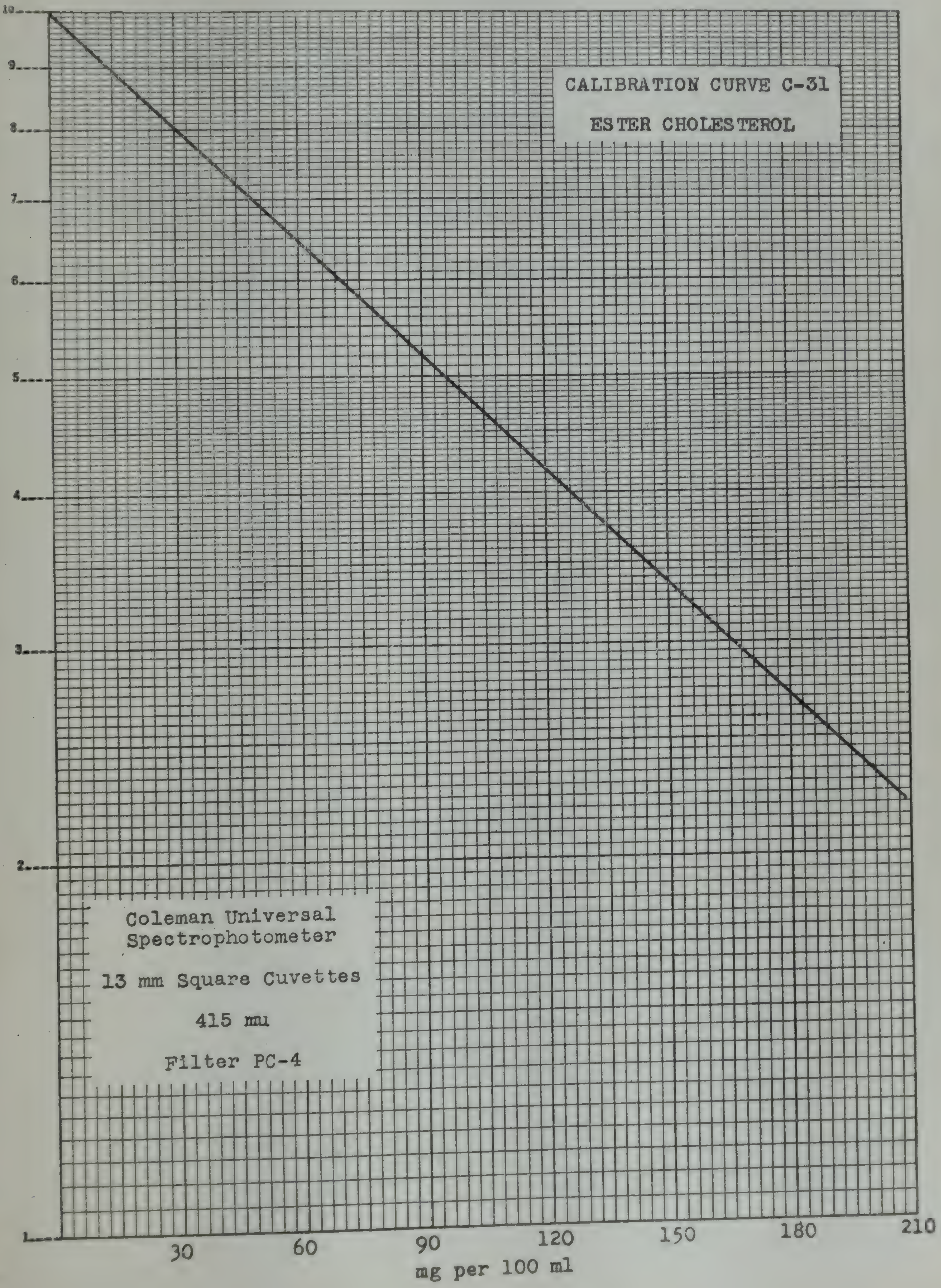
CALIBRATION CURVE C-31
ESTER CHOLESTEROL
Coleman Universal Spectrophotometer
13 mm Square Cuvettes
415 mu
Filter PC-4
10
9
8
7
6
5
4
3
2
1
30
60
90
120
150
180
210
mg per 100 ml

Meter Reading	0	1	2	3	4	5	6	7	8	9
0										
10										
20										
30										
40										
50										
60										
70										
80										
90										

CHOLESTEROL, TOTAL

A spectrophotometric adaptation based on the methods and modifications of Bernoulli (1932), Obermer and Milton (1933; 1937), Schoenheimer and Sperry (1934), Sperry (1938), Rose, Schattner and Exton (1939; 1941) and Sobel and Mayer (1945)

PRINCIPLE

Total cholesterol is extracted and proteins are precipitated simultaneously by means of an acetone : alcohol mixture. After evaporation of the solvent, the residue is taken up with alcohol and saponified to convert the cholesterol esters to the free form. The total free cholesterol is precipitated by digitonin; the cholesteryl digitonide is dissolved in acetic acid and treated with zinc and acetyl chlorides to produce a rose-red color (Tschugaew reaction).

DETERMINATION IN PLASMA OR SERUM

SAMPLE

Serum or heparinized plasma from blood withdrawn during post-absorptive state—analyses preferably made on plasma. Whole blood and oxalated plasma should not be used. The plasma or serum must be separated from the red cells as soon as possible. The determination of total cholesterol may be made any time within 24 hours after withdrawal of the blood.

TEST PROCEDURE

Special Apparatus

Fister cholesterol tubes—15 ml; glass-stoppered; the 5, 7, 10 and 12 ml graduations must be accurately calibrated.

Ostwald-Van Slyke micro pipettes accurately calibrated "to deliver" 0.2 ml.

Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.

Note: All apparatus should be thoroughly oven-dried before use.

Reagents

1. **Acetone:Alcohol Mixture, 1:1 (Schoenheimer-Sperry).** Mix equal volumes of redistilled acetone ($(CH_3)_2CO$—Baker & Adamson, A.C.S.) and redistilled absolute ethyl alcohol (C_2H_5OH—U.S. Industrial Chemicals, U.S.P.). Preserve with adequate precautions against absorption of moisture.
2. **Ethyl Alcohol, 95%** (C_2H_5OH—U.S. Industrial Chemicals, U.S.P.). Redistilled.
3. **Sodium Ethylate, 15%.** Add 8.6 gm of small, freshly cut pieces of bright metallic sodium (Na—Baker & Adamson, A.C.S.) to 100 ml absolute ethyl alcohol (C_2H_5OH—U.S. Industrial Chemicals, U.S.P.), adding one piece at a time and waiting until each piece has completely reacted before adding another; cool to room temperature and adjust to a volume of 100 ml with absolute ethyl alcohol; filter through a Schleicher & Schuell #576 paper. Prepare in small quantities and discard when a brownish color develops. Preserve in Pyrex glass-stoppered bottle, the stopper and neck of which are well waxed to prevent freezing.
4. **Hydrochloric Acid Solution.** Dilute 160 ml concentrated hydrochloric acid (HCl—Baker & Adamson, A.C.S.) to 1000 ml with distilled water; titrate against the 15% sodium ethylate (3) using phenolphthalein as indicator and adjust so that 1 ml of the ethylate requires 2 ml of the acid for neutralization; check by re-titration. Preserve in Pyrex glass-stoppered bottle.
5. **Digitonin, 1% Alcoholic.** Dissolve 1 gm recrystallized digitonin ($C_{54}H_{92}O_{28}$—Reagent Chemicals, Special) in 100 ml 50% ethyl alcohol (a) at 60° C.; allow to stand for 24 hours and filter off any precipitate that may have formed. The filtrate should be crystal clear and remains stable for at least six months. Note: Since the cholesteryl digitonide may vary with different preparations of digitonin and thus alter the chromogenic response of the color reagents, the calibration curve should be checked for each new lot of digitonin.
   (a) Ethyl alcohol, 50% aqueous; in a 100 ml mixing cylinder place 55 ml 95% ethyl alcohol (2) and dilute to volume with distilled water; allow to cool and adjust volume.
6. **Acetone:Ether Wash Solution, 1:2 (Schoenheimer-Sperry).** Mix 1 volume redistilled acetone ($(CH_3)_2CO$—Baker & Adamson, A.C.S.) and 2 volumes anhydrous peroxide-free ether (Reagent #7).
7. **Ether, Anhydrous, Peroxide-Free** ($(C_2H_5)_2O$—Mallinckrodt, AR). All samples of ether should be checked for the presence of peroxide as follows: Shake a small sample of ether with one-half volume freshly prepared 10% potassium iodide, slightly acidified with concentrated sulfuric acid; if more than a very faint yellow color develops, the ether must be purified by washing with 10% sodium sulfite followed by several washings with distilled water, and distilled over calcium chloride. Preserve in the dark in a tightly stoppered brown glass bottle containing a coil of pure iron wire.
8. **Zinc Chloride Reagent (Rose-Schattner-Exton).** Place 38 gm anhydrous fused zinc chloride ($ZnCl_2$—Baker & Adamson, Reagent, Fused Sticks) in a 250 ml Erlenmeyer flask; heat over a free flame until all moisture and fumes are driven

off and a clear melt results; rotate the flask so that the melt is evenly distributed over the sides of the lower half of the flask; allow to cool to less than 100° C.; add 50 ml glacial acetic acid (CH_3COOH—Baker & Adamson, A.C.S.) and heat gently until solution is complete; transfer to a 100 ml mixing cylinder; rinse out the flask with small portions of acetic acid and transfer the rinsings to the cylinder; dilute to 100 ml with acetic acid. Preserve in glass-stoppered bottle with adequate precautions against absorption of moisture.

9. **Acetyl Chloride** (CH_3COCl—J. T. Baker, CP Analyzed).

Technic

Note: For concentrations of total cholesterol in excess of 350 mg per 100 ml, use 0.1 ml sample in Step 1 and multiply the values obtained from the calibration table by 2. It is essential that moisture be excluded throughout the procedure.

1. In a special dry glass-stoppered cholesterol tube place
   0.2 ml sample from an Ostwald-Van Slyke micro pipette.
2. Add 10 ml acetone:alcohol mixture (1), delivered rapidly in a fine stream directly onto the sample.
3. Tightly stopper the tube and place in a water bath at 80° C. for 10 minutes, shaking frequently and vigorously—the stopper may be carefully removed occasionally during the heating period to relieve the pressure, precautions being taken to prevent any water from entering the tube.
4. Remove the tube from the water bath and cool to room temperature by placing in a beaker half-filled with cold water.
5. Adjust the volume to exactly 10 ml with acetone:-alcohol mixture (1); stopper and mix thoroughly.
6. Centrifuge at 2000 rpm for 10 minutes or until the supernatant fluid is clear.
7. Transfer 4 ml clear centrifugate to another special dry glass-stoppered cholesterol tube (sample).
8. Remove the acetone:alcohol by aeration or by placing the tube in a water bath at 70° C. until completely dry.
9. Add 1 ml 95% ethyl alcohol (2) and dissolve the residue by gently rotating the tube.
10. Add 0.1 ml 15% sodium ethylate (3), stopper and mix by gently rotating the tube.
11. Place tube in water bath at 55° C. for 60 minutes.
12. Cool to room temperature by placing in cold water.
13. Add 0.25 ml hydrochloric acid solution (4) and mix by gently rotating the tube.
14. Add 0.5 ml 1% digitonin (5), delivered in a rapid stream while constantly agitating the tube.
15. Stopper the tube and place in incubator at 37° C. for 2 hours—longer does no harm.
16. Gently stir the solution with a thin glass rod to free any particles that may have adhered to the walls of the tube near the surface of the liquid; carefully remove the rod without touching the upper part of the tube and place on a small pipette rest so that no adherent precipitate is lost.
17. Centrifuge at 1500 rpm for 3 minutes and then at 2500 rpm for 7 minutes.
18. Carefully decant and discard the supernatant fluid and allow the tube to drain in an inverted position for 3 minutes, wiping off the lip of the tube with clean tissue.
19. Replace the rod in the tube and add from a pipette 5 ml acetone:ether wash solution (6), washing down the rod and the walls of the tube with the final portion of solution.
20. Remove the rod, place on rest as before, and repeat Steps 17 and 18.
21. Replace the rod in the tube and add from a pipette 5 ml ether (7), washing down the rod and the walls of the tube with the final portion of solution.
22. Remove the rod, place on rest as before, and repeat Steps 17 and 18.
23. Replace the rod in the tube and place in water bath at 40° C. until the last trace of ether has disappeared—the rod is allowed to remain in the tube through Step 27.
24. Add 6 ml zinc chloride reagent (8) from a burette and mix well with rod.
25. Add 4 ml acetyl chloride (9) from a burette and mix well with rod.
26. In another special dry glass-stoppered cholesterol tube (reagent blank) place
    6 ml zinc chloride reagent (8)
    4 ml acetyl chloride (9), stopper and mix by inversion.
27. Immediately immerse both tubes in water bath at 55° C.; stir the contents of the sample tube vigorously with the rod until the precipitate is completely dissolved, remove rod and stopper at once—the level of the water in the bath should not be above the shoulders of the tubes.
28. Allow to remain in water bath for 15 minutes—a rose-red color develops in the sample tube.
29. Without removing the stoppers, cool to room temperature by placing in cold water.
30. Wipe all the water off the outside of the tubes and transfer to correspondingly marked cuvettes.
31. Measure the transmittancy of the sample against the reagent blank set at 100% transmission at a wave length of **515** mμ and obtain the concentration of total cholesterol from the calibration table.

CALIBRATION PROCEDURE

Special Apparatus

Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.

Fister cholesterol tubes—15 ml; glass-stoppered; the

5, 7, 10 and 12 ml graduations must be accurately calibrated.

Reagents

2. **Ethyl Alcohol, 95%.** Same as for Test Procedure.
3. **Sodium Ethylate, 15%.** Same as for Test Procedure.
4. **Hydrochloric Acid Solution.** Same as for Test Procedure.
5. **Digitonin, 1% Alcoholic.** Same as for Test Procedure.
6. **Acetone:Ether Wash Solution, 1:2 (Schoenheimer-Sperry).** Same as for Test Procedure.
7. **Ether, Anhydrous, Peroxide-Free.** Same as for Test Procedure.
8. **Zinc Chloride Reagent (Rose-Schattner-Exton).** Same as for Test Procedure.
9. **Acetyl Chloride.** Same as for Test Procedure.
10. **Total Cholesterol Standards**

Note: When preparing any standard with alcohol as a solvent, note the temperature and always bring the standard to this temperature before use.

(a) Cholesterol stock standard (1 ml contains 1 mg cholesterol). In a 100 ml volumetric flask place 100 mg recrystallized free cholesterol ($C_{27}H_{45}OH$—Reagent Chemicals, Special); dissolve in 95% ethyl alcohol (2) and dilute to volume with same. Preserve in a glass-stoppered bottle.

(b) Dilute standard (1 ml contains 0.4 mg cholesterol). In a 50 ml volumetric flask place 20 ml stock standard (10a) and dilute to volume with 95% ethyl alcohol (2). Prepare day of use and keep in a glass-stoppered bottle.

(c) Calibration standards. In a series of seven accurately calibrated 10 ml volumetric flasks place respectively 0, 1, 2, 3, 4, 5 and 8 ml of the dilute standard (10b) and dilute to volume with 95% ethyl alcohol (2). These standards represent concentrations of total cholesterol equivalent to 0, 50, 100, 150, 200, 250 and 400 mg per 100 ml sample. Prepare just before use.

Technic

1. Transfer 1 ml of each calibration standard (10c) to a correspondingly marked special dry glass-stoppered cholesterol tube.
2. Proceed exactly as outlined in the Test Procedure, Steps 10–30 inclusive, omitting Step 26 since this duplicates the zero concentration standard.
3. Set the zero concentration standard at 100% transmission at a wave length of **515** mμ and record the transmittancy of the 50, 100, 150, 200, 250 and 400 mg per cent standards. The transmittancy of the 400 mg standard should be approximately 24%.
4. Plot the observed values on semi-log graph paper—the points fall on a straight line which passes through the origin (Calibration Curve C-32).
5. Prepare a calibration table from the T-C values obtained from the plotted curve.

PROCEDURE FOR USE WITH LINEAR-SCALE PHOTOELECTRIC COLORIMETERS (SHEARD-SANFORD TYPE)

TEST PROCEDURE

Exactly as outlined; set the reagent blank at 100 and use a green filter.

CALIBRATION PROCEDURE

Exactly as outlined—use a green filter; plot the observed readings on semi-log graph paper—the points fall on a straight line passing through the origin.

PROCEDURE FOR USE WITH LOGARITHMIC-SCALE PHOTOELECTRIC COLORIMETERS (KLETT-SUMMERSON TYPE)

TEST PROCEDURE

Exactly as outlined; set the reagent blank at 0 and use a green filter.

CALIBRATION PROCEDURE

Exactly as outlined—use a green filter; plot the observed readings on linear graph paper—the points fall on a straight line passing through the origin.

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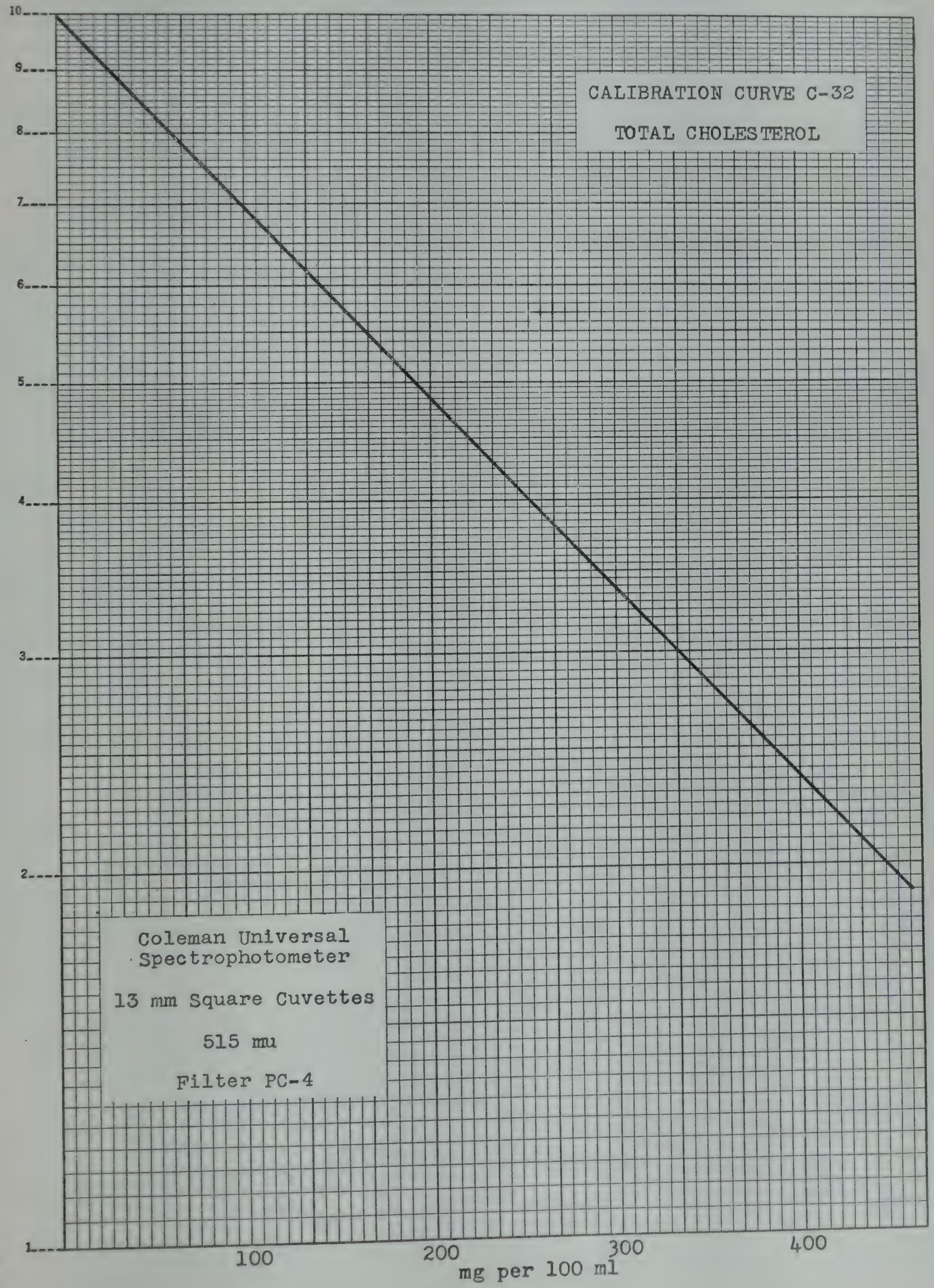
NOTES


CALIBRATION CURVE C-32
TOTAL CHOLESTEROL
Coleman Universal Spectrophotometer
13 mm Square Cuvettes
515 mμ
Filter PC-4
10
9
8
7
6
5
4
3
2
1
100
200
300
400
mg per 100 ml

Meter Reading	0	1	2	3	4	5	6	7	8	9
0										
10										
20										
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40										
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70										
80										
90										

CHOLESTEROL, FREE AND ESTER

A spectrophotometric adaptation based on the methods and modifications of Bernoulli (1932), Obermer and Milton (1933; 1937), Schoenheimer and Sperry (1934), Sperry (1938), Rose, Schattner and Exton (1939; 1941) and Sobel and Mayer (1945)

PRINCIPLE

Total cholesterol is extracted and proteins are precipitated simultaneously by means of an acetone:alcohol mixture. After evaporation of the solvent, the residue is taken up with alcohol and the free cholesterol precipitated by digitonin without previous saponification. The analysis is then carried out as outlined in the method for total cholesterol (Method C-32), the same analytical principles being involved. Ester cholesterol is the difference between the total cholesterol and the free cholesterol.

DETERMINATION IN PLASMA OR SERUM

SAMPLE

Serum or heparinized plasma from blood withdrawn during post-absorptive state—analyses preferably made on plasma. Whole blood and oxalated plasma should not be used. The plasma or serum must be separated from the red cells as soon as possible. The determination of the free fraction must be started within 30 minutes after withdrawal of the blood and carried through the digitonin precipitation without delay.

TEST PROCEDURE

Special Apparatus

Fister cholesterol tubes—15 ml; glass-stoppered; the 5, 7, 10 and 12 ml graduations must be accurately calibrated.

Ostwald-Van Slyke micro pipettes accurately calibrated "to deliver" 0.2 ml.

Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.

Note: All apparatus should be thoroughly oven-dried before use.

Reagents

1. **Acetone:Alcohol Mixture, 1:1 (Schoenheimer-Sperry).** Mix equal volumes of redistilled acetone (($(CH_3)_2CO$—Baker & Adamson, A.C.S.) and redistilled absolute ethyl alcohol (C_2H_5OH—U. S. Industrial Chemicals, U.S.P.). Preserve with adequate precautions against absorption of moisture.
2. **Ethyl Alcohol, 95%** (C_2H_5OH—U.S. Industrial Chemicals, U.S.P.). Redistilled.
3. **Digitonin, 1% Alcoholic.** Dissolve 1 gm recrystallized digitonin ($C_{54}H_{92}O_{28}$—Reagent Chemicals, Special) in 100 ml 50% ethyl alcohol (a) at 60° C.; allow to stand for 24 hours and filter off any precipitate that may have formed. The filtrate should be crystal clear and remains stable for at least six months. Note: Since the cholesteryl digitonide may vary with different preparations of digitonin and thus alter the chromogenic response of the color reagents, the calibration curve should be checked for each new lot of digitonin.
    (a) Ethyl alcohol, 50% aqueous; in a 100 ml mixing cylinder place 55 ml 95% ethyl alcohol (2) and dilute to volume with distilled water; allow to cool and adjust volume.
4. **Acetone:Ether Wash Solution, 1:2 (Schoenheimer-Sperry).** Mix 1 volume redistilled acetone (($(CH_3)_2CO$—Baker & Adamson, A.C.S.) and 2 volumes anhydrous peroxide-free ether (Reagent #5).
5. **Ether, Anhydrous, Peroxide-Free** (($(C_2H_5)_2O$—Mallinckrodt, AR). All samples of ether should be checked for the presence of peroxide as follows: shake a small sample of ether with one-half volume freshly prepared 10% potassium iodide slightly acidified with concentrated sulfuric acid; if more than a very faint yellow color develops, the ether must be purified by washing with 10% sodium sulfite followed by several washings with distilled water, and distilled over calcium chloride. Preserve in the dark in a tightly stoppered brown glass bottle containing a coil of pure iron wire.
6. **Zinc Chloride Reagent (Rose-Schattner-Exton).** Place 33 gm anhydrous fused zinc chloride ($ZnCl_2$—Baker & Adamson, Reagent, Fused Sticks) in a 250 ml Erlenmeyer flask; heat over a free flame until all moisture and fumes are driven off and a clear melt results; rotate the flask so that the melt is evenly distributed over the sides of the lower half of the flask; allow to cool to less than 100° C.; add 50 ml glacial acetic acid (CH_3COOH—Baker & Adamson, A.C.S.) and heat gently until solution is complete; transfer to a 100 ml mixing cylinder; rinse out the flask with small portions of acetic acid and transfer the rinsings to the cylinder; dilute to 100 ml with acetic acid. Preserve in glass-stoppered bottle with adequate precautions against absorption of moisture.
7. **Acetyl Chloride** (CH_3COCl—J. T. Baker, CP Analyzed).

Technic

Note: It is essential that moisture be excluded throughout the procedure.

1. In a special dry glass-stoppered cholesterol tube place
   0.2 ml sample from an Ostwald-Van Slyke micro pipette.
2. Add 10 ml acetone: alcohol mixture (1), delivered rapidly in a fine stream directly onto the sample.
3. Tightly stopper the tube and place in a water bath at 80° C. for 10 minutes, shaking frequently and vigorously—the stopper may be carefully removed occasionally during the heating period to relieve the pressure, precautions being taken to prevent any water from entering the tube.
4. Remove the tube from the water bath and cool to room temperature by placing in a beaker half-filled with cold water.
5. Adjust the volume to exactly 10 ml with acetone: alcohol mixture (1); stopper and mix thoroughly.
6. Centrifuge at 2000 rpm for 10 minutes or until the supernatant fluid is clear.
7. Transfer 4 ml clear centrifugate to another special dry glass-stoppered cholesterol tube (sample).
8. Remove the acetone: alcohol by aeration or by placing the tube in a water bath at 70° C. until completely dry.
9. Add 1 ml 95% ethyl alcohol (2) and dissolve the residue by gently rotating the tube.
10. Add 0.5 ml 1% digitonin (3), delivered in a rapid stream while constantly agitating the tube.
11. Stopper the tube and place in incubator at 37° C. for 2 hours—longer does no harm.
12. Gently stir the solution with a thin glass rod to free any particles that may have adhered to the walls of the tube near the surface of the liquid; carefully remove the rod without touching the upper part of the tube and place on a small pipette rest so that no adherent precipitate is lost.
13. Centrifuge at 1500 rpm for 3 minutes and then at 2500 rpm for 7 minutes.
14. Carefully decant and discard the supernatant fluid and allow the tube to drain in an inverted position for 3 minutes, wiping off the lip of the tube with clean tissue.
15. Replace the rod in the tube and add from a pipette 5 ml acetone: ether wash solution (4), washing down the rod and the walls of the tube with the final portion of solution.
16. Remove the rod, place on rest as before, and repeat Steps 13 and 14.
17. Replace the rod in the tube and add from a pipette 5 ml ether (5), washing down the rod and the walls of the tube with the final portion of solution.
18. Remove the rod, place on rest as before, and repeat Steps 13 and 14.
19. Replace the rod in the tube and place in water bath at 40° C. until the last trace of ether has disappeared—the rod is allowed to remain in the tube through Step 23.
20. Add 3 ml zinc chloride reagent (6) from a burette and mix well with rod.
21. Add 2 ml acetyl chloride (7) from a burette and mix well with rod.
22. In another special dry glass-stoppered cholesterol tube (reagent blank) place
    3 ml zinc chloride reagent (6)
    2 ml acetyl chloride (7), stopper and mix by inversion.
23. Immediately immerse both tubes in water bath at 55° C.; stir the contents of the sample tube vigorously with the rod until the precipitate is completely dissolved, remove rod and stopper at once —the level of the water in the bath should not be above the shoulders of the tubes.
24. Allow to remain in water bath for 15 minutes—a rose-red color develops in the sample tube.
25. Without removing the stoppers, cool to room temperature by placing in cold water.
26. Wipe all the water off the outside of the tubes and transfer to correspondingly marked cuvettes.
27. Measure the transmittancy of the sample against the reagent blank set at 100% transmission at a wave length of **515** mμ and divide the value from the calibration table for total cholesterol (Method C-32) by 2 to obtain the concentration of free cholesterol in mg per 100 ml sample.

Calculation

Total cholesterol in mg per 100 ml − Free cholesterol in mg per 100 ml = Ester cholesterol in mg per 100 ml

Note: The total cholesterol concentration is determined as outlined in Method C-32.

CALIBRATION PROCEDURE

This calibration procedure need not be carried out. The concentration of free cholesterol is obtained by dividing the values from the calibration table for total cholesterol (Method C-32) by 2.

PROCEDURE FOR USE WITH LINEAR-SCALE PHOTOELECTRIC COLORIMETERS (SHEARD-SANFORD TYPE)

TEST PROCEDURE

Exactly as outlined; set the reagent blank at 100 and use a green filter.

CALIBRATION PROCEDURE

Need not be performed; the concentration of free cholesterol is obtained by dividing the values from the calibration table for total cholesterol (Method C-32) by 2.

PROCEDURE FOR USE WITH LOGARITHMIC-SCALE PHOTOELECTRIC COLORIMETERS (KLETT-SUMMERSON TYPE)

TEST PROCEDURE

Exactly as outlined; set the reagent blank at 0 and use a green filter.

CALIBRATION PROCEDURE

Need not be performed; the concentration of free cholesterol is obtained by dividing the values from the calibration table for total cholesterol (Method C-32) by 2.

BIBLIOGRAPHY

Same as listed under total cholesterol (Method C-32).

NOTES

NOTES

COPPER

A spectrophotometric adaptation based on the methods and modifications of Warburg (1927), Callan and Henderson (1929), Locke, Main and Rosbash (1932), McFarlane (1932), Tompsett (1934), Sachs, Levine, Andersen and Schmit (1941) and Cartwright, Jones and Wintrobe (1945).

PRINCIPLE

Copper reacts with sodium diethyl dithiocarbamate to form a golden brown copper salt, interference by iron being eliminated by the use of pyrophosphate. The color produced by normal amounts of copper is too pale to permit accurate measurement in the range of greatest importance and therefore a known amount of copper is added to the unknown to enhance the color intensity. Extraction with amyl alcohol further concentrates and intensifies the color.

DETERMINATION IN WHOLE BLOOD, PLASMA OR SERUM

SAMPLE

Blood withdrawn during post-absorptive state. Anticoagulant for whole blood or plasma—potassium oxalate. Analyses preferably made on whole blood.

TEST PROCEDURE

Special Apparatus

Centrifuge tubes, Pyrex, 15 ml.

Ostwald-Folin pipettes accurately calibrated "to contain" 5 ml.

Graduated test tubes—25 ml in 0.5 ml; the 15 ml graduation must be accurately calibrated.

Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.

Note: Glassware must be thoroughly cleaned and rinsed at least three times with copper-free distilled water (Reagent #1).

Reagents

1. **Water, Distilled, Copper-Free.** Redistil distilled water from an all-glass Pyrex distillation apparatus. This must be used for the preparation of all reagents and standards and where indicated in the test procedure.
2. **Trichloracetic Acid, 20% Aqueous.** Distil trichloracetic acid (CCl_3COOH—J. T. Baker, CP Analyzed) in vacuo; dissolve 20 gm in copper-free distilled water (1) and dilute to 100 ml. Preserve in Pyrex glass-stoppered bottle.
3. **Copper Standard (1 ml contains 0.005 mg).** In a 50 ml volumetric flask place 25 ml of the dilute standard (Reagent #8b) and dilute to volume with copper-free distilled water (1). Prepare fresh daily.
4. **Sodium Pyrophosphate, Saturated Aqueous.** Add 50 gm crystalline sodium pyrophosphate ($Na_4P_2O_7 \cdot 10H_2O$—Baker & Adamson, Reagent) to 100 ml copper-free distilled water (1); heat to boiling and allow to cool; use supernatant fluid.
5. **Ammonium Hydroxide, Concentrated, 28%.** Distill ammonium hydroxide (NH_4OH—Baker & Adamson, A.C.S.) from an all-glass distillation apparatus into copper-free distilled water (1) until solution is saturated with ammonia gas. Preserve in resistant-glass bottle.
6. **Sodium Diethyl Dithiocarbamate, 0.1% Aqueous.** Dissolve 100 mg sodium diethyl dithiocarbamate ($(C_2H_5)_2NCSSNa$—Eastman #2596) in copper-free distilled water (1) and dilute to 100 ml. Preserve in brown glass bottle. Stability several weeks.
7. **iso-Amyl Alcohol** ($C_5H_{11}OH$—Baker & Adamson, A.C.S.).

Technic

1. In a 15 ml Pyrex centrifuge tube place 1 ml copper-free distilled water (1).
2. Add 5 ml sample from an Ostwald-Folin pipette, thoroughly rinsing out the pipette by drawing in and expelling some of the mixture several times.
3. Mix with a thin glass rod, allowing the rod to remain in the tube.
4. Place the tube in boiling water and allow to remain until the liquid becomes opaque.
5. Cool under running tap water for 3 minutes.
6. Add 2 ml 20% trichloracetic acid (2) and stir thoroughly—do not remove rod from tube.
7. Heat in water bath at 90–95° C. for 5 minutes, stirring frequently.
8. Cool under running tap water for 3 minutes.
9. Remove rod, washing off with a few ml of copper-free distilled water (1).
10. Centrifuge at 3000 rpm for 10 minutes.
11. Decant the supernatant fluid into a 25 ml graduated test tube (sample).
12. To the original centrifuge tube add
    1 ml 20% trichloracetic acid (2)
    1 ml copper-free distilled water (1).
13. Break up the precipitate thoroughly with the same glass rod used previously and repeat Steps 7–10 inclusive.
14. Decant the supernatant fluid into the test tube containing the first decantation.
15. Repeat Steps 12–14 inclusive.
16. To the combined centrifugates add 1 ml copper standard (3) and mix.

17. In another 25 ml graduated test tube (reagent blank) place
    - 4 ml 20% trichloracetic acid (2)
    - 7 ml copper-free distilled water (1).
18. To each tube add
    - 1 ml saturated sodium pyrophosphate (4) and mix
    - 2 ml ammonium hydroxide (5) and mix
    - 1 ml 0.1% sodium diethyl dithiocarbamate (6) and mix.
19. Add exactly 6 ml iso-amyl alcohol (7).
20. Stopper the tubes and shake vigorously for 15 seconds—the alcohol layer is now colored a golden brown.
21. Allow the layers to separate for a few minutes and then carefully transfer the alcohol layer, by means of a capillary pipette with rubber bulb, onto a dry 7 cm Whatman #40 paper and dry funnel and allow to drain into a dry cuvette.
22. Immediately measure the transmittancy of the sample against the reagent blank set at 100% transmission at a wave length of 440 mμ and multiply the value from the calibration table by 20 to obtain the concentration of copper in gamma per 100 ml sample.

CALIBRATION PROCEDURE

Special Apparatus

Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.

Graduated test tubes—25 ml in 0.5 ml; the 15 ml graduation must be accurately calibrated.

Reagents

1. **Water, Distilled, Copper-Free.** Same as for Test Procedure.
2. **Trichloracetic Acid, 20% Aqueous.** Same as for Test Procedure.
3. **Copper Standard (1 ml contains 0.005 mg).** Same as for Test Procedure.
4. **Sodium Pyrophosphate, Saturated Aqueous.** Same as for Test Procedure.
5. **Ammonium Hydroxide, Concentrated, 28%.** Same as for Test Procedure.
6. **Sodium Diethyl Dithiocarbamate, 0.1% Aqueous.** Same as for Test Procedure.
7. **iso-Amyl Alcohol.** Same as for Test Procedure.
8. **Copper Standards**
    - (a) Copper stock standard (1 ml contains 1 mg copper). In a 100 ml volumetric flask place 392.8 mg crystalline copper sulfate ($CuSO_4 \cdot 5H_2O$—Reagent Chemicals, Special); add about 50 ml copper-free distilled water (1) and shake until dissolved; dilute to volume with same. It is essential to use crystals showing no sign of efflorescence.
    - (b) Dilute standard (1 ml contains 0.01 mg copper). In a 100 ml volumetric flask place 1 ml stock standard (8a) and dilute to volume with copper-free distilled water (1). Prepare just before use.
    - (c) Calibration standards. In a series of five accurately calibrated 100 ml volumetric flasks place respectively 0, 5, 10, 15 and 20 ml of the dilute standard (8b); dilute to volume with copper-free distilled water (1). These standards contain concentrations of copper equivalent to 0, 2.5, 5, 7.5 and 10 gamma per 5 ml. Prepare just before use.

Technic

Note: Unlike the other procedures in this manual, the calibration curve is plotted and the calibration table is constructed on the copper concentration in the volume of standard used in order that more accurate readings may be made in the normal range.

1. Place 5 ml of each calibration standard (8c) into a correspondingly marked 25 ml graduated test tube.
2. To each tube add 4 ml 20% trichloracetic acid (2) and mix.
3. Add 1 ml copper standard (3) and 1 ml copper-free distilled water (1).
4. Proceed exactly as outlined in the Test Procedure, Steps 17–21 inclusive.
5. Set the reagent blank at 100% transmission at a wave length of 440 mμ and record the transmittancy of the 0, 2.5, 5.0, 7.5 and 10 gamma standards. The transmittancy of the zero concentration standard should be approximately 76% and that of the 10 gamma standard approximately 43%.
6. Plot the observed values on semi-log graph paper—the points fall on a straight line which passes through the transmission axis near 76% transmission (Calibration Curve C-35). Note: Because of the addition of a known quantity of copper to each calibration standard, the calibration curve does not pass through the origin; since the same amount of copper is added to the unknown, correction for this increment is unnecessary.
7. Prepare a calibration table from the T-C values obtained from the plotted curve.

PROCEDURE FOR USE WITH LINEAR-SCALE PHOTOELECTRIC COLORIMETERS (SHEARD-SANFORD TYPE)

TEST PROCEDURE

Exactly as outlined; set the reagent blank at 100 and use a blue filter.

CALIBRATION PROCEDURE

Exactly as outlined—use a blue filter; plot the observed readings on semi-log graph paper—the points fall on a straight line passing through the transmission axis.

PROCEDURE FOR USE WITH LOGARITHMIC-SCALE PHOTOELECTRIC COLORIMETERS (KLETT-SUMMERSON TYPE)

TEST PROCEDURE

Exactly as outlined; set the reagent blank at 0 and use a blue filter.

CALIBRATION PROCEDURE

Exactly as outlined—use a blue filter; plot the observed readings on linear graph paper—the points fall on a straight line passing through the instrument reading axis.

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NOTES

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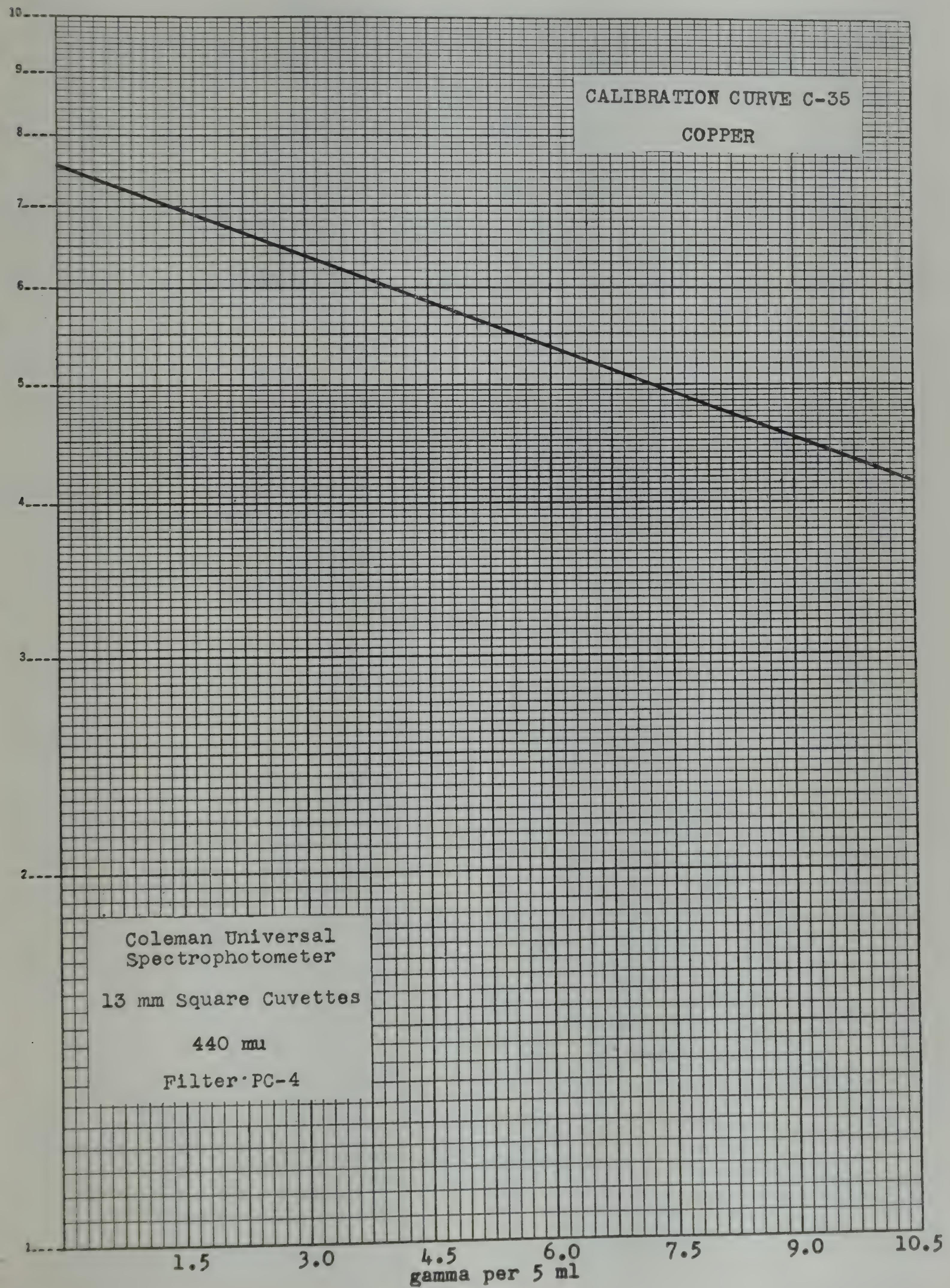
CALIBRATION CURVE C-35
COPPER
Coleman Universal Spectrophotometer
13 mm Square Cuvettes
440 mμ
Filter PC-4
10
9
8
7
6
5
4
3
2
1
1.5
3.0
4.5
6.0
7.5
9.0
10.5
gamma per 5 ml

Meter Reading	0	1	2	3	4	5	6	7	8	9
0										
10										
20										
30										
40										
50										
60										
70										
80										
90										

CREATINE

A spectrophotometric adaptation based on the methods and modifications of Pitts (1934), Bolliger (1936), Benedict and Behre (1936), Langley and Evans (1936), Andes (1938), Peters (1942) and Jansen, Sombroek and Noyons (1947)

PRINCIPLE

Creatine is hydrolyzed to creatinine which, together with the preformed creatinine is termed "total creatinine." Creatine, expressed as creatinine, is the difference between the total creatinine and the preformed creatinine. The analytical principles involved are the same as for the determination of preformed creatinine (Method C-42).

DETERMINATION IN WHOLE BLOOD, PLASMA OR SERUM

SAMPLE

Blood withdrawn during post-absorptive state. Anticoagulant for whole blood or plasma—potassium oxalate. Analyses preferably made on plasma or serum. Preformed creatinine must be determined on a similar sample.

TEST PROCEDURE

Special Apparatus

Ostwald-Folin pipettes accurately calibrated "to contain" 4 ml.
Graduated test tubes—10 ml in 0.1 ml; the 5 ml graduation must be accurately calibrated.
Glass spheres or balls to loosely fit the mouths of the above tubes if hydrolysis procedure (A) is followed,
or
Tin foil, C.P. if hydrolysis procedure (B) is followed.
Capillary droppers delivering 40 drops per ml.
Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.

Reagents

1. **Sulfuric Acid, Volumetric 4N/3.** Slowly add 40 ml concentrated sulfuric acid (H_2SO_4—Grasselli, A.C.S.) to 1000 ml distilled water and mix well; titrate against standard 1 N alkali using phenolphthalein as indicator; adjust accordingly and re-titrate—20 ml of 4N/3 acid require 26.7 ml of 1 N alkali.
2. **Sodium Tungstate, 20% Aqueous.** Dissolve 20 gm sodium tungstate ($Na_2WO_4 \cdot 2H_2O$—Mallinckrodt, Folin Special) in distilled water and dilute to 100 ml. This solution must be neutral or slightly alkaline to phenolphthalein, not more than 0.8 ml being required to neutralize 10 ml of 0.1 N hydrochloric acid. Preserve in resistant-glass bottle.
3. **Hydrochloric Acid, Volumetric 3 N.** Add 270 ml concentrated hydrochloric acid (HCl—Baker & Adamson, A.C.S.) to 1000 ml distilled water and mix well; titrate against standard 1 N alkali using phenolphthalein as indicator; adjust accordingly and re-titrate—10 ml of 3 N acid require 30 ml of 1 N alkali. Required only if hydrolysis procedure (A) is followed.
4. **Sodium Hydroxide, Volumetric 3 N.** Prepare by diluting slightly more than the required amount of concentrated carbonate-free sodium hydroxide solution (see Appendix) and titrate against standard 1 N acid using phenolphthalein as indicator; adjust accordingly and retitrate—40 ml of 1 N acid require 13.3 ml of 3 N alkali. Preserve in ceresin-lined bottle.
5. **3,5-Dinitrobenzoic Acid Reagent (Langley-Evans).** Place 10 gm recrystallized 3,5-dinitrobenzoic acid ($(NO_2)_2C_6H_3COOH$—Reagent Chemicals, Special, according to Jansen, Sombroek and Noyons) in a 250 ml beaker; pulverize with a footed glass rod; add slowly and with constant stirring 25 ml 10% sodium carbonate (a); add 75 ml distilled water; heat until solution is complete—do not boil; allow to cool to room temperature; adjust volume to 100 ml with distilled water; filter. The color of the reagent should be no more than a pale yellow—if deeper, discard. A satisfactory reagent should show no tinge of red or brown and remain practically colorless when 0.8 ml of the reagent, 2 ml distilled water and 0.4 ml 1 N sodium hydroxide are mixed.
   (a) Sodium carbonate, 10% aqueous; dissolve 10 gm anhydrous sodium carbonate (Na_2CO_3—Baker & Adamson, A.C.S.) in distilled water and dilute to 100 ml; if not clear, filter. Preserve in ceresin-lined bottle.

Technic

Note: For concentrations of total creatinine in excess of 10 mg per 100 ml, dilute 2.5 ml of the protein-free centrifugate with an equal volume of distilled water in Step 7 and multiply the values obtained from the calibration table for preformed creatinine (Method C-42) by 2.

1. In a 50 ml Erlenmeyer flask place
   12 ml distilled water.
2. Add 4 ml sample from an Ostwald-Folin pipette, thoroughly rinsing out the pipette by drawing in and expelling some of the mixture several times.
3. Mix the contents of the flask by gentle rotation until hemolysis is complete.
4. Add 2 ml 4N/3 sulfuric acid (1), drop by drop, while constantly but gently, rotating the flask; mix by gentle rotation until the fluid is uniformly black-brown.
5. Add 2 ml 20% sodium tungstate (2), drop by drop, while constantly but gently, rotating the flask; shake vigorously until the mixture is chocolate colored and foam-free.
6. Transfer the contents of the flask to a 50 ml centrifuge tube and centrifuge at 1500 rpm for 5 minutes.

Either one of the following methods of hydrolysis may be employed.

(A)

7. In a 10 ml graduated test tube (total creatinine) place
   5 ml clear protein-free centrifugate.
8. Add 1 ml 3 N hydrochloric acid (3).
9. Cover the mouth of the tube with a loose-fitting glass sphere.
10. Immerse the tube in boiling water for 4 hours.
11. Cool under running tap water to room temperature.
12. Add 1 ml 3 N sodium hydroxide (4).
13. Adjust volume to exactly 5 ml with distilled water and proceed with Step 14.

(B)

7. In a 10 ml graduated test tube (total creatinine) place
   5 ml clear protein-free centrifugate.
8. Cover the mouth of the tube with tin foil.
9. Place the tube in an autoclave and heat at 130° C. for 20 minutes (or at 155° C. for 10 minutes).
10. Cool under running tap water to room temperature.
11. Adjust volume to exactly 5 ml with distilled water and proceed with Step 14.

From this point on, the procedure is identical with either method of hydrolysis.

14. In a second 10 ml graduated test tube (preformed creatinine) place
    5 ml clear protein-free centrifugate.
15. In a third 10 ml graduated test tube (reagent blank) place
    5 ml distilled water.
16. To each tube add 1 drop 3 N sodium hydroxide (4) from a capillary-tipped dropper.
17. Add 1.5 ml 3,5-dinitrobenzoic acid reagent (5) and mix.
18. Add 0.25 ml 3 N sodium hydroxide (4) and mix.
19. Allow to stand in the dark at room temperature for 10 minutes.
20. Measure the transmittancy of the total creatinine and the preformed creatinine against the reagent blank set at 100% transmission at a wave length of 495 mμ and obtain the concentrations from the calibration table for preformed creatinine (Method C-42).

Calculation

Total creatinine in mg per 100 ml − Preformed creatinine in mg per 100 ml = Creatine (in terms of creatinine) in mg per 100 ml

Creatine (in terms of creatinine) in mg per 100 ml × 1.159 = Creatine in mg per 100 ml

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CALIBRATION PROCEDURE

This calibration procedure need not be carried out. The values for total creatinine and preformed creatinine are obtained directly from the calibration table for preformed creatinine (Method C-42).

Method C-40b

DETERMINATION IN URINE

SAMPLE

Aliquot of a 24-hour specimen. Keep cold during collection.

TEST PROCEDURE

Special Apparatus

As listed under the Test Procedure for blood.

Reagents

1. **Sulfuric Acid, Volumetric 4N/3.** Same as for blood.
2. **Sodium Tungstate, 20% Aqueous.** Same as for blood.
3. **Hydrochloric Acid, Volumetric 3 N.** Same as for blood.

4. **Sodium Hydroxide, Volumetric 3 N.** Same as for blood.
5. **3,5-Dinitrobenzoic Acid Reagent (Langley-Evans).** Same as for blood.

Technic

Note: For pale and dilute samples, dilute 10 ml to 100 ml in Step 2 and multiply the values obtained from the calibration table for preformed creatinine (Method C-42) by 10; for dark and concentrated samples, dilute 2.5 ml to 100 ml in Step 2 and multiply the values obtained from the calibration table by 40.

1. Filter a small portion of well mixed urine.
2. In a 100 ml volumetric flask place 5 ml filtered urine and dilute to volume with distilled water.
3. Treat 4 ml diluted urine exactly as outlined in the Test Procedure for blood Steps 1–19 inclusive.
4. Measure the transmittancy of the total creatinine and the preformed creatinine against the reagent blank set at 100% transmission at a wave length of 495 mμ and multiply the values from the calibration table for preformed creatinine (Method C-42) by 20 to obtain the concentrations of total creatinine and preformed creatinine in mg per 100 ml urine.

Calculation

Total creatinine in mg per 100 ml − Preformed creatinine in mg per 100 ml = Creatine (in terms of creatinine) in mg per 100 ml

Creatine (in terms of creatinine) in mg per 100 ml × 1.159 = Creatine in mg per 100 ml

$$\frac{\text{Volume of 24-hour specimen}}{100} \times \text{Creatine in mg per 100 ml} = \text{Mg creatine excreted in 24 hours}$$

PROCEDURE FOR USE WITH LINEAR-SCALE PHOTOELECTRIC COLORIMETERS (SHEARD-SANFORD TYPE)

TEST PROCEDURE

Exactly as outlined; set the reagent blank at 100 and use a blue filter.

CALIBRATION PROCEDURE

Need not be performed; the concentrations of total creatinine and preformed creatinine are obtained directly from the calibration table for preformed creatinine (Method C-42).

PROCEDURE FOR USE WITH LOGARITHMIC-SCALE PHOTOELECTRIC COLORIMETERS (KLETT-SUMMERSON TYPE)

TEST PROCEDURE

Exactly as outlined; set the reagent blank at 0 and use a blue filter.

CALIBRATION PROCEDURE

Need not be performed; the concentrations of total creatinine and preformed creatinine are obtained directly from the calibration table for preformed creatinine (Method C-42).

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NOTES

CREATINE

A spectrophotometric adaptation based on the methods and modifications of Folin and Wu (1919), Pitts (1934), Hoffman (1941), Peters (1942) and Phillips (1946)

PRINCIPLE

Creatine is hydrolyzed to creatinine which, together with the preformed creatinine is termed "total creatinine." Creatine, expressed as creatinine, is the difference between the total creatinine and the preformed creatinine. The analytical principles involved are the same as for the determination of preformed creatinine (Method C-43).

DETERMINATION IN WHOLE BLOOD, PLASMA OR SERUM

SAMPLE

Blood withdrawn during post-absorptive state. Anticoagulant for whole blood or plasma—potassium oxalate. Analyses preferably made on plasma or serum. Preformed creatinine must be determined on a similar sample.

TEST PROCEDURE

Special Apparatus

Ostwald-Folin pipettes accurately calibrated "to contain" 2 ml.

Graduated test tubes—10 ml in 0.1 ml; the 5 ml graduation must be accurately calibrated.

Glass spheres or balls to loosely fit the mouths of the above tubes if hydrolysis procedure (A) is followed,

or

Tin foil, C.P. if hydrolysis procedure (B) is followed.

Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.

Reagents

1. **Sulfuric Acid, Volumetric N/12.** Add 2.5 ml concentrated sulfuric acid (H_2SO_4—Grasselli, A.C.S.) to 1000 ml distilled water and mix well; titrate against standard 0.1 N alkali using phenolphthalein as indicator; adjust accordingly and re-titrate—20 ml of N/12 acid require 16.7 ml of 0.1 N alkali.
2. **Sodium Tungstate, 10% Aqueous.** Dissolve 10 gm sodium tungstate ($Na_2WO_4\cdot 2H_2O$—Mallinckrodt, Folin Special) in distilled water and dilute to 100 ml. The solution must be neutral or slightly alkaline to phenolphthalein, not more than 0.4 ml being required to neutralize 10 ml 0.1 N hydrochloric acid. Preserve in resistant-glass bottle.
3. **Hydrochloric Acid, Volumetric 3 N.** Add 270 ml concentrated hydrochloric acid (HCl—Baker & Adamson, A.C.S.) to 1000 ml distilled water and mix well; titrate against standard 1 N alkali using phenolphthalein as indicator; adjust accordingly and re-titrate—10 ml of 3 N acid require 30 ml of 1 N alkali. Required only if hydrolysis procedure (A) is followed.
4. **Sodium Hydroxide, Volumetric 3 N.** Prepare by diluting slightly more than the required amount of concentrated carbonate-free sodium hydroxide solution (see Appendix) and titrate against standard 1 N acid using phenolphthalein as indicator; adjust accordingly and re-titrate—40 ml of 1 N acid require 13.3 ml of 3 N alkali. Preserve in ceresin-lined bottle. Required only if hydrolysis procedure (A) is followed.
5. **Alkaline Picrate Reagent (Phillips).** In a 25 ml mixing cylinder place 5 ml 1% picric acid (a) and 2.5 ml 2.5 N sodium hydroxide (b); dilute to volume with distilled water and mix by inversion. Use within 5 minutes after preparation.
    - (a) Picric acid, 1% aqueous; dissolve 12 gm picric acid ($HO\cdot C_6H_2\cdot(NO_2)_3$—J. T. Baker, Special for Blood Test) in distilled water and dilute to 1000 ml; titrate 20 ml against standard 0.1 N alkali using phenolphthalein as indicator—20 ml of the picric acid solution must require 8.61–8.81 ml of 0.1 N alkali; adjust accordingly and re-titrate. Preserve in brown glass bottle.
    - (b) Sodium hydroxide, volumetric 2.5 N; prepare by diluting slightly more than the required amount of concentrated carbonate-free sodium hydroxide solution (see Appendix); dilute 20 ml of this approximately 2.5 N solution to 100 ml; titrate against standard 0.5 N acid using phenolphthalein as indicator; adjust accordingly and re-titrate—20 ml of 0.5 N acid require 20 ml of a 1:5 dilution of 2.5 N alkali. Preserve in ceresin-lined bottle.

Technic

Note: For concentrations of total creatinine in excess of 10 mg per 100 ml, dilute 2.5 ml of the protein-free centrifugate with an equal volume of distilled water in Step 7 and multiply the values obtained from the calibration table for preformed creatinine (Method C-43) by 2.

1. In a 50 ml Erlenmeyer flask place
   16 ml N/12 sulfuric acid (1).

2. Add 2 ml sample from an Ostwald-Folin pipette, thoroughly rinsing out the pipette by drawing in and expelling some of the mixture several times.
3. Mix the contents of the flask by gentle rotation until the fluid is uniformly black-brown.
4. Add 2 ml 10% sodium tungstate (2), drop by drop, while constantly but gently, rotating the flask.
5. Shake the flask vigorously until the mixture is chocolate colored and foam-free.
6. Transfer the contents of the flask to a 50 ml centrifuge tube and centrifuge at 1500 rpm for 5 minutes.

Either one of the following methods of hydrolysis may be employed.

(A)

7. In a 10 ml graduated test tube (total creatinine) place
   5 ml clear protein-free centrifugate.
8. Add 1 ml 3 N hydrochloric acid (3).
9. Cover the mouth of the tube with a loose-fitting glass sphere.
10. Immerse the tube in boiling water for 4 hours.
11. Cool under running tap water to room temperature.
12. Add 1 ml 3 N sodium hydroxide (4).
13. Adjust volume to exactly 5 ml with distilled water and proceed with Step 14.

(B)

7. In a 10 ml graduated test tube (total creatinine) place
   5 ml clear protein-free centrifugate.
8. Cover the mouth of the tube with tin foil.
9. Place the tube in an autoclave and heat at 130° C. for 20 minutes (or at 155° C. for 10 minutes).
10. Cool under running tap water to room temperature.
11. Adjust volume to exactly 5 ml with distilled water and proceed with Step 14.

From this point on, the procedure is identical with either method of hydrolysis.

14. In a second 10 ml graduated test tube (preformed creatinine) place
    5 ml clear protein-free centrifugate.
15. In a third 10 ml graduated test tube (reagent blank) place
    5 ml distilled water.
16. To each tube add 2.5 ml freshly prepared alkaline picrate reagent (5).
17. Place the tubes in water bath at 25° C. for 60 minutes.
18. Measure the transmittancy of the total creatinine and the preformed creatinine against the reagent blank set at 100% transmission at a wave length of 520 mμ and obtain the concentrations from the calibration table for preformed creatinine (Method C-43).

Calculation

Total creatinine in mg per 100 ml − Preformed creatinine in mg per 100 ml = Creatine (in terms of creatinine) in mg per 100 ml

Creatine (in terms of creatinine) in mg per 100 ml × 1.159 = Creatine in mg per 100 ml

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CALIBRATION PROCEDURE

This calibration procedure need not be carried out. The values for total creatinine and preformed creatinine are obtained directly from the calibration table for preformed creatinine (Method C-43).

Method C-41b

DETERMINATION IN URINE

SAMPLE

Aliquot of a 24-hour specimen. Keep cold during collection.

TEST PROCEDURE

Special Apparatus

As listed under the Test Procedure for blood.

Reagents

1. **Sulfuric Acid, Volumetric N/12.** Same as for blood.
2. **Sodium Tungstate, 10% Aqueous.** Same as for blood.
3. **Hydrochloric Acid, Volumetric 3 N.** Same as for blood.
4. **Sodium Hydroxide, Volumetric 3 N.** Same as for blood.

5. **Alkaline Picrate Reagent (Phillips).** Same as for blood.

Technic

Note: For pale and dilute samples, dilute 10 ml to 100 ml in Step 2 and multiply the values obtained from the calibration table for preformed creatinine (Method C-43) by 10; for dark and concentrated samples, dilute 2.5 ml to 100 ml in Step 2 and multiply the values obtained from the calibration table by 40.

1. Filter a small portion of well mixed urine.
2. In a 100 ml volumetric flask place 5 ml filtered urine and dilute to volume with distilled water.
3. Treat 2 ml diluted urine exactly as outlined in the Test Procedure for blood Steps 1–17 inclusive.
4. Measure the transmittancy of the total creatinine and the preformed creatinine against the reagent blank set at 100% transmission at a wave length of 520 mμ and multiply the values from the calibration table for preformed creatinine (Method C-43) by 20 to obtain the concentrations of total creatinine and preformed creatinine in mg per 100 ml urine.

Calculation

$$\text{Total creatinine in mg per 100 ml} - \text{Preformed creatinine in mg per 100 ml} = \text{Creatine (in terms of creatinine) in mg per 100 ml}$$

$$\text{Creatine (in terms of creatinine) in mg per 100 ml} \times 1.159 = \text{Creatine in mg per 100 ml}$$

$$\frac{\text{Volume of 24-hour specimen}}{100} \times \text{Creatine in mg per 100 ml} = \text{Mg creatine excreted in 24 hours}$$

PROCEDURE FOR USE WITH LINEAR-SCALE PHOTOELECTRIC COLORIMETERS (SHEARD-SANFORD TYPE)

TEST PROCEDURE

Exactly as outlined; set the reagent blank at 100 and use a green filter.

CALIBRATION PROCEDURE

Need not be performed; the concentrations of total creatinine and preformed creatinine are obtained directly from the calibration table for preformed creatinine (Method C-43).

PROCEDURE FOR USE WITH LOGARITHMIC-SCALE PHOTOELECTRIC COLORIMETERS (KLETT-SUMMERSON TYPE)

TEST PROCEDURE

Exactly as outlined; set the reagent blank at 0 and use a green filter.

CALIBRATION PROCEDURE

Need not be performed; the concentrations of total creatinine and preformed creatinine are obtained directly from the calibration table for preformed creatinine (Method C-43).

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NOTES

CREATININE, PREFORMED

A spectrophotometric adaptation based on the methods and modifications of Bolliger (1936), Benedict and Behre (1936), Langley and Evans (1936), Andes (1938) and Jansen, Sombroek and Noyons (1947)

PRINCIPLE

Creatinine in alkaline solution, upon treatment with the sodium salt of 3,5-dinitrobenzoic acid, produces a garnet red color (Bolliger reaction).

DETERMINATION IN WHOLE BLOOD, PLASMA OR SERUM

SAMPLE

Blood withdrawn during post-absorptive state. Anticoagulant for whole blood or plasma—potassium oxalate. Analyses preferably made on plasma.

TEST PROCEDURE

Special Apparatus

Ostwald-Folin pipettes accurately calibrated "to contain" 2 ml.

Capillary droppers delivering 40 drops per ml.

Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.

Reagents

1. **Sulfuric Acid, Volumetric 4N/3.** Slowly add 40 ml concentrated sulfuric acid (H_2SO_4—Grasselli, A.C.S.) to 1000 ml distilled water and mix well; titrate against standard 1 N alkali using phenolphthalein as indicator; adjust accordingly and re-titrate—20 ml of 4N/3 acid require 26.7 ml of 1 N alkali.
2. **Sodium Tungstate, 20% Aqueous.** Dissolve 20 gm sodium tungstate ($Na_2WO_4 \cdot 2H_2O$—Mallinckrodt, Folin Special) in distilled water and dilute to 100 ml. This solution must be neutral or slightly alkaline to phenolphthalein, not more than 0.8 ml being required to neutralize 10 ml of 0.1 N hydrochloric acid. Preserve in resistant-glass bottle.
3. **Sodium Hydroxide, Volumetric 2.5 N.** Prepare by diluting slightly more than the required amount of concentrated carbonate-free sodium hydroxide solution (see Appendix); dilute 20 ml of this approximately 2.5 N solution to 100 ml; titrate against standard 0.5 N acid using phenolphthalein as indicator; adjust accordingly and re-titrate—20 ml of 0.5 N acid require 20 ml of a 1:5 dilution of 2.5 N alkali. Preserve in ceresin-lined bottle.
4. **3,5-Dinitrobenzoic Acid Reagent (Langley-Evans).** Place 10 gm recrystallized 3,5-dinitrobenzoic acid ($(NO_2)_2C_6H_3COOH$—Reagent Chemicals, Special, according to Jansen, Sombroek and Noyons) in a 250 ml beaker; pulverize with a footed glass rod; add slowly and with constant stirring 25 ml 10% sodium carbonate (a); add 75 ml distilled water; heat until solution is complete—do not boil; allow to cool to room temperature; adjust volume to 100 ml with distilled water; filter. The color of the reagent should be no more than a pale yellow—if deeper, discard. A satisfactory reagent should show no tinge of red or brown and remain practically colorless when 0.8 ml of the reagent, 2 ml distilled water and 0.4 ml 1 N sodium hydroxide are mixed.
   - (a) Sodium carbonate, 10% aqueous; dissolve 10 gm anhydrous sodium carbonate (Na_2CO_3—Baker & Adamson, A.C.S.) in distilled water and dilute to 100 ml; if not clear, filter. Preserve in ceresin-lined bottle.

Technic

Note: For concentrations of creatinine in excess of 10 mg per 100 ml, dilute 2.5 ml of the protein-free centrifugate with an equal volume of distilled water in Step 7 and multiply the values obtained from the calibration table by 2.

1. In a 25 ml Erlenmeyer flask place
   6 ml distilled water.
2. Add 2 ml sample from an Ostwald-Folin pipette, thoroughly rinsing out the pipette by drawing in and expelling some of the mixture several times.
3. Mix the contents of the flask by gentle rotation until hemolysis is complete.
4. Add 1 ml 4N/3 sulfuric acid (1), drop by drop, while constantly but gently, rotating the flask; mix by gentle rotation until the fluid is uniformly black-brown.
5. Add 1 ml 20% sodium tungstate (2), drop by drop, while constantly but gently, rotating the flask; shake vigorously until the mixture is chocolate colored and foam-free.
6. Transfer the contents of the flask to a 15 ml centrifuge tube and centrifuge at 1500 rpm for 5 minutes.
7. In a cuvette (sample) place
   5 ml clear protein-free centrifugate.
8. In another cuvette (reagent blank) place
   5 ml distilled water.
9. To each cuvette add 1 drop 2.5 N sodium hydroxide (3) from a capillary-tipped dropper.
10. Add 1.5 ml 3,5-dinitrobenzoic acid reagent (4) and mix.
11. Add 0.25 ml 2.5 N sodium hydroxide (3) and mix.
12. Allow to stand in the dark at room temperature for 10 minutes.

13. Measure the transmittancy of the sample against the reagent blank set at 100% transmission at a wave length of **495** mμ and obtain the concentration of creatinine from the calibration table.

CALIBRATION PROCEDURE

Special Apparatus

Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.
Capillary droppers delivering 40 drops per ml.

Reagents

3. **Sodium Hydroxide, Volumetric 2.5 N.** Same as for Test Procedure.
4. **3,5-Dinitrobenzoic Acid Reagent (Langley-Evans).** Same as for Test Procedure.
5. **Hydrochloric Acid, Volumetric 0.1 N.** Add 10 ml concentrated hydrochloric acid (HCl—Baker & Adamson, A.C.S.) to 1000 ml distilled water and mix; standardize against 0.1 N alkali using phenolphthalein as indicator.
6. **Creatinine Standards**
   (a) Creatinine stock standard (1 ml contains 1 mg creatinine). In a 100 ml volumetric flask place 160.24 mg creatinine zinc chloride ($(C_4H_7N_3O)_2 \cdot ZnCl_2$—Reagent Chemicals, Special); dissolve in 0.1 N hydrochloric acid (5) and dilute to volume with same. Preserve with a few drops of toluene. Stability indefinite.
   (b) Dilute standard (1 ml contains 0.02 mg creatinine). In a 100 ml volumetric flask place 2 ml stock standard (6a) and dilute to volume with distilled water. Prepare the day of use.
   (c) Calibration standards. In a series of seven accurately calibrated 10 ml volumetric flasks place respectively 0, 1, 2, 4, 6, 8 and 10 ml of the dilute standard (6b) and dilute to volume with distilled water. These standards represent concentrations of creatinine equivalent to 0, 1, 2, 4, 6, 8 and 10 mg per 100 ml sample. Prepare just before use.

Technic

1. Transfer 5 ml of each calibration standard (6c) to a correspondingly marked cuvette and proceed exactly as outlined in the Test Procedure, Steps 9–12 inclusive.
2. Set the zero concentration standard, which should show no pink or reddish tinge, at 100% transmission at a wave length of **495** mμ and record the transmittancy of the 1, 2, 4, 6, 8 and 10 mg per cent standards. The transmittancy of the 10 mg standard should be approximately 35%.
3. Plot the observed values on semi-log graph paper—the points fall on a straight line which passes through the origin (Calibration Curve C-42).
4. Prepare a calibration table from the T-C values obtained from the plotted curve.

Method C-42b

DETERMINATION IN URINE

SAMPLE

Aliquot of a 24-hour specimen. Keep cold during collection.

TEST PROCEDURE

Special Apparatus

As listed under the Test Procedure for blood.

Reagents

1. **Sulfuric Acid, Volumetric 4N/3.** Same as for blood.
2. **Sodium Tungstate, 20% Aqueous.** Same as for blood.
3. **Sodium Hydroxide, Volumetric 2.5 N.** Same as for blood.
4. **3,5-Dinitrobenzoic Acid Reagent (Langley-Evans).** Same as for blood.

Technic

Note: For pale and dilute samples, dilute 10 ml to 100 ml in Step 2 and multiply the values obtained from the calibration table for blood by 10; for dark and concentrated samples, dilute 2.5 ml to 100 ml in Step 2 and multiply the values obtained from the calibration table by 40.

1. Filter a small portion of well mixed urine.
2. In a 100 ml volumetric flask place 5 ml filtered urine and dilute to volume with distilled water.
3. Treat 2 ml diluted urine exactly as outlined in the Test Procedure for blood Steps 1–12 inclusive.

4. Measure the transmittancy of the sample against the reagent blank set at 100% transmission at a wave length of **495** mμ and multiply the value from the calibration table for blood by 20 to obtain the concentration of creatinine in mg per 100 ml urine.

Calculation

$$\frac{\text{Volume of 24-hour specimen}}{100} \times \text{Creatinine in mg per 100 ml} = \text{Mg creatinine excreted in 24 hours}$$

PROCEDURE FOR USE WITH LINEAR-SCALE PHOTOELECTRIC COLORIMETERS (SHEARD-SANFORD TYPE)

TEST PROCEDURE

Exactly as outlined; set the reagent blank at 100 and use a blue filter.

CALIBRATION PROCEDURE

Exactly as outlined—use a blue filter; plot the observed readings on semi-log graph paper—the points fall on a straight line passing through the origin.

PROCEDURE FOR USE WITH LOGARITHMIC-SCALE PHOTOELECTRIC COLORIMETERS (KLETT-SUMMERSON TYPE)

TEST PROCEDURE

Exactly as outlined; set the reagent blank at 0 and use a blue filter.

CALIBRATION PROCEDURE

Exactly as outlined—use a blue filter; plot the observed readings on linear graph paper—the points fall on a straight line passing through the origin.

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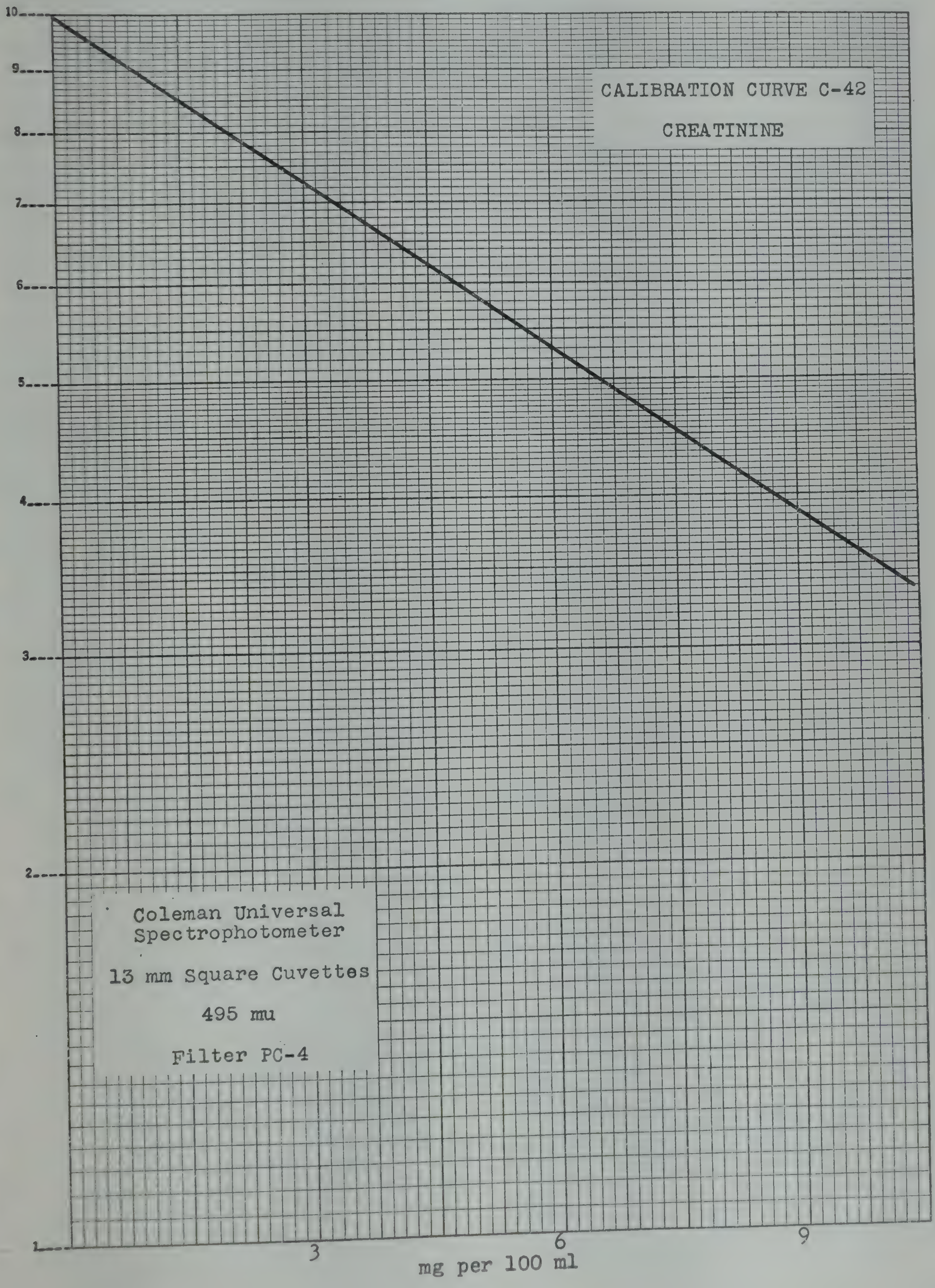
CALIBRATION CURVE C-42
CREATININE
Coleman Universal Spectrophotometer
13 mm Square Cuvettes
495 mu
Filter PC-4
10
9
8
7
6
5
4
3
2
1
3
6
9
mg per 100 ml

Meter Reading	0	1	2	3	4	5	6	7	8	9
0										
10										
20										
30										
40										
50										
60										
70										
80										
90										

CREATININE, PREFORMED

A spectrophotometric adaptation based on the methods and modifications of Folin and Wu (1919), Hoffman (1941), Peters (1942) and Phillips (1946)

PRINCIPLE

Creatinine in a protein-free centrifugate is treated with picric acid in alkaline solution to produce a deep red tautomer of creatinine picrate (Jaffe reaction).

DETERMINATION IN WHOLE BLOOD, PLASMA OR SERUM

SAMPLE

Blood withdrawn during post-absorptive state. Anticoagulant for whole blood or plasma—potassium oxalate. Analyses preferably made on plasma or serum.

TEST PROCEDURE

Special Apparatus

Ostwald-Folin pipettes accurately calibrated "to contain" 1 ml.
Graduated test tubes—10 ml in 0.1 ml.
Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.

Reagents

1. **Sulfuric Acid, Volumetric N/12.** Add 2.5 ml concentrated sulfuric acid (H_2SO_4—Grasselli, A.C.S.) to 1000 ml distilled water and mix well; titrate against standard 0.1 N alkali using phenolphthalein as indicator; adjust accordingly and re-titrate—20 ml of N/12 acid require 16.7 ml of 0.1 N alkali.
2. **Sodium Tungstate, 10% Aqueous.** Dissolve 10 gm sodium tungstate ($Na_2WO_4 \cdot 2H_2O$—Mallinckrodt, Folin Special) in distilled water and dilute to 100 ml. The solution must be neutral or slightly alkaline to phenolphthalein, not more than 0.4 ml being required to neutralize 10 ml 0.1 N hydrochloric acid. Preserve in resistant-glass bottle.
3. **Alkaline Picrate Reagent (Phillips).** In a 25 ml mixing cylinder place 5 ml 1% picric acid (a) and 2.5 ml 2.5 N sodium hydroxide (b); dilute to volume with distilled water and mix by inversion. Use within 5 minutes after preparation.
    (a) Picric acid, 1% aqueous; dissolve 12 gm picric acid ($HO \cdot C_6H_2 \cdot (NO_2)_3$—J. T. Baker, Special for Blood Test) in distilled water and dilute to 1000 ml; titrate 20 ml against standard 0.1 N alkali using phenolphthalein as indicator—20 ml of the picric acid solution must require 8.61–8.81 ml of 0.1 N alkali; adjust accordingly and re-titrate. Preserve in brown glass bottle.
    (b) Sodium hydroxide, volumetric 2.5 N; prepare by diluting slightly more than the required amount of concentrated carbonate-free sodium hydroxide solution (see Appendix); dilute 20 ml of this approximately 2.5 N solution to 100 ml; titrate against standard 0.5 N acid using phenolphthalein as indicator; adjust accordingly and re-titrate—20 ml of 0.5 N acid require 20 ml of a 1:5 dilution of 2.5 N alkali. Preserve in ceresin-lined bottle.

Technic

Note: For concentrations of creatinine in excess of 10 mg per 100 ml, dilute 2.5 ml of the protein-free centrifugate with an equal volume of distilled water in Step 7 and multiply the values obtained from the calibration table by 2.

1. In a 25 ml Erlenmeyer flask place
   8 ml N/12 sulfuric acid (1).
2. Add 1 ml sample from an Ostwald-Folin pipette, thoroughly rinsing out the pipette by drawing in and expelling some of the mixture several times.
3. Mix the contents of the flask by gentle rotation until the fluid is uniformly black-brown.
4. Add 1 ml 10% sodium tungstate (2), drop by drop, while constantly but gently, rotating the flask.
5. Shake the flask vigorously until the mixture is chocolate colored and foam-free.
6. Transfer the contents of the flask to a 15 ml centrifuge tube and centrifuge at 1500 rpm for 5 minutes.
7. In a 10 ml graduated test tube (sample) place
   5 ml clear protein-free centrifugate.
8. In another 10 ml graduated test tube (reagent blank) place
   5 ml distilled water.
9. To each tube add 2.5 ml freshly prepared alkaline picrate reagent (3) and mix.
10. Place the tubes in water bath at 25° C. for 60 minutes.
11. Measure the transmittancy of the sample against the reagent blank set at 100% transmission at a wave length of 520 mμ and obtain the concentration of creatinine from the calibration table.

CALIBRATION PROCEDURE

Special Apparatus

Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.
Graduated test tubes—10 ml in 0.1 ml.

Reagents

3. **Alkaline Picrate Reagent (Phillips).** Same as for Test Procedure.

4. **Hydrochloric Acid, Volumetric 0.1 N.** Add 10 ml concentrated hydrochloric acid (HCl—Baker & Adamson, A.C.S.) to 1000 ml distilled water and mix; standardize against 0.1 N alkali using phenolphthalein as indicator.

5. **Creatinine Standards**
   (a) Creatinine stock standard (1 ml contains 1 mg creatinine). In a 100 ml volumetric flask place 160.24 mg creatinine zinc chloride ($(C_4H_7N_3O)_2 \cdot ZnCl_2$—Reagent Chemicals, Special); dissolve in 0.1 N hydrochloric acid (4) and dilute to volume with same. Preserve with a few drops of toluene. Stability indefinite.
   (b) Dilute standard (1 ml contains 0.01 mg creatinine). In a 100 ml volumetric flask place 1 ml stock standard (5a) and dilute to volume with distilled water. Prepare the day of use.
   (c) Calibration standards. In a series of seven accurately calibrated 10 ml volumetric flasks place respectively 0, 1, 2, 4, 6, 8 and 10 ml of the dilute standard (5b) and dilute to volume with distilled water. These standards represent concentrations of creatinine equivalent to 0, 1, 2, 4, 6, 8 and 10 mg per 100 ml sample. Prepare just before use.

Technic

1. Transfer 5 ml of each calibration standard (5c) to a correspondingly marked 10 ml graduated test tube.
2. Proceed exactly as outlined in the Test Procedure, Steps 9–10 inclusive.
3. Set the zero concentration standard at 100% transmission at a wave length of **520** mμ and record the transmittancy of the 1, 2, 4, 6, 8 and 10 mg per cent standards. The transmittancy of the 10 mg standard should be approximately 32.5%.
4. Plot the observed values on semi-log graph paper—the points fall on a slight curve which passes through the origin (Calibration Curve C-43).
5. Prepare a calibration table from the T-C values obtained from the plotted curve.

Method C-43b

DETERMINATION IN URINE

SAMPLE

Aliquot of a 24-hour specimen. Keep cold during collection.

TEST PROCEDURE

Special Apparatus

As listed under the Test Procedure for blood.

Reagents

1. **Sulfuric Acid, Volumetric N/12.** Same as for blood.
2. **Sodium Tungstate, 10% Aqueous.** Same as for blood.
3. **Alkaline Picrate Reagent** (Phillips). Same as for blood.

Technic

Note: For pale and dilute samples, dilute 10 ml to 100 ml in Step 2 and multiply the values obtained from the calibration table for blood by 10; for dark and concentrated samples, dilute 2.5 ml to 100 ml in Step 2 and multiply the values obtained from the calibration table by 40.

1. Filter a small portion of well mixed urine.
2. In a 100 ml volumetric flask place 5 ml filtered urine and dilute to volume with distilled water.
3. Treat 1 ml diluted urine exactly as outlined in the Test Procedure for blood Steps 1–10 inclusive.
4. Measure the transmittancy of the sample against the reagent blank set at 100% transmission at a wave length of **520** mμ and multiply the value from the calibration table for blood by 20 to obtain the concentration of creatinine in mg per 100 ml urine.

Calculation

$$\frac{\text{Volume of 24-hour specimen}}{100} \times \text{Creatinine in mg per 100 ml} = \text{Mg creatinine excreted in 24 hours}$$

PROCEDURE FOR USE WITH LINEAR-SCALE PHOTOELECTRIC COLORIMETERS (SHEARD-SANFORD TYPE)

TEST PROCEDURE

Exactly as outlined; set the reagent blank at 100 and use a green filter.

CALIBRATION PROCEDURE

Exactly as outlined—use a green filter; plot the observed readings on semi-log graph paper—the points fall on a slight curve passing through the origin.

PROCEDURE FOR USE WITH LOGARITHMIC-SCALE PHOTOELECTRIC COLORIMETERS (KLETT-SUMMERSON TYPE)

TEST PROCEDURE

Exactly as outlined; set the reagent blank at 0 and use a green filter.

CALIBRATION PROCEDURE

Exactly as outlined—use a green filter; plot the observed readings on linear graph paper—the points fall on a slight curve passing through the origin.

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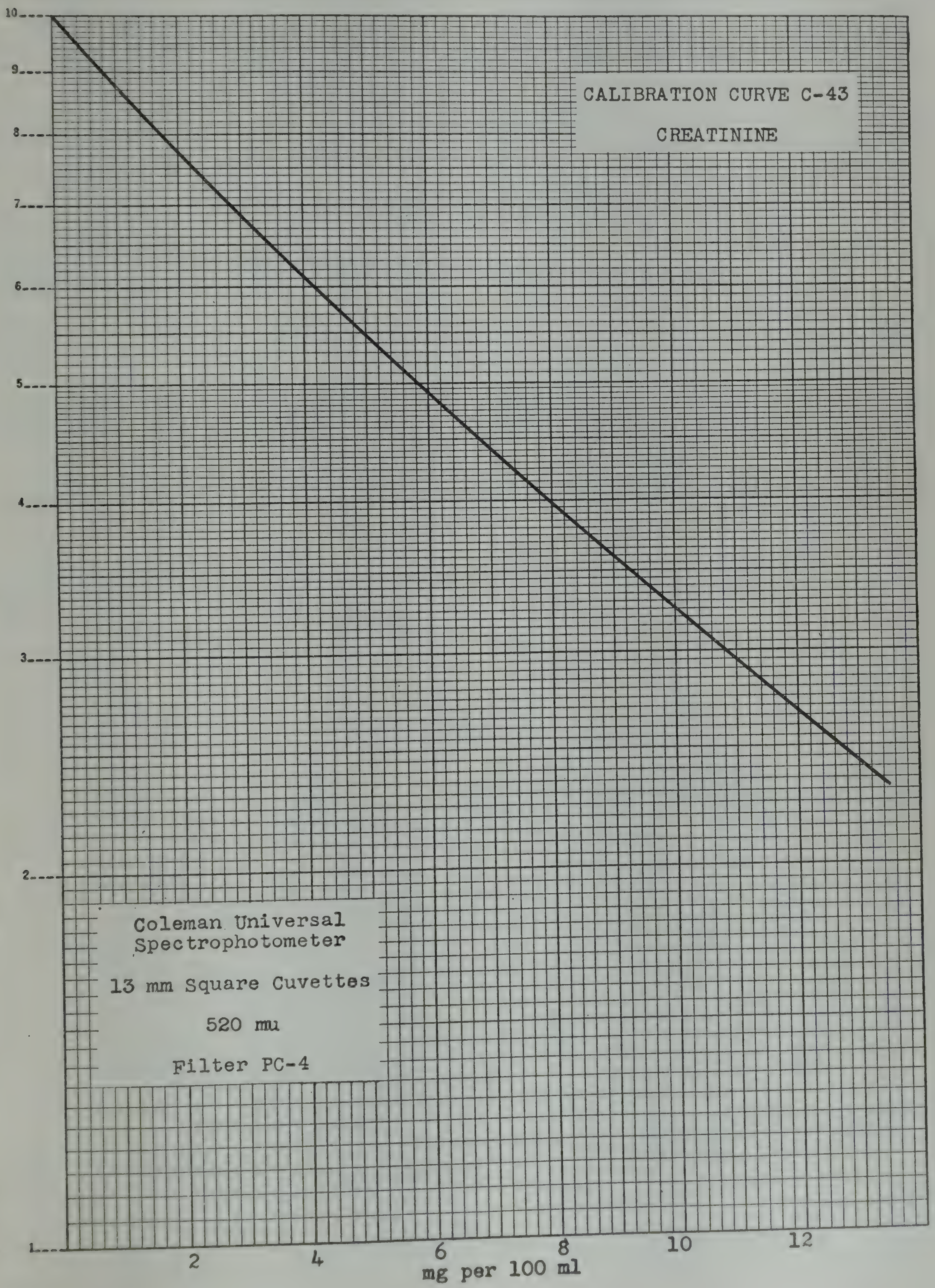
CALIBRATION CURVE C-43
CREATININE
Coleman Universal Spectrophotometer
13 mm Square Cuvettes
520 mu
Filter PC-4
10
9
8
7
6
5
4
3
2
1
2
4
6
8
10
12
mg per 100 ml

Meter Reading	0	1	2	3	4	5	6	7	8	9
0										
10										
20										
30										
40										
50										
60										
70										
80										
90										

"DEXTROSE"
TOTAL REDUCING SUBSTANCES

A spectrophotometric adaptation based on the methods and modifications of
Folin and Wu (1919; 1920), Byrd (1927), Folin (1929), Sahyun (1931; 1936)
and Fiorentino and Giannettasio (1940)

PRINCIPLE

Dextrose, as well as other substances in the blood, reduces copper in an alkaline tartrate solution to cuprous oxide which in turn acts as a reducing agent on phosphomolybdic acid to form blue phosphomolybdous acid (Folin-Wu reaction), the color of which is dependent upon the amount of cuprous oxide and consequently directly proportional to the original concentration of reducing agents. The phosphomolybdic acid also destroys the excess color due to the copper reagent. The use of the special alkaline copper tartrate and phosphomolybdic acid reagents yields a final color of marked stability.

DETERMINATION IN WHOLE BLOOD, PLASMA OR SERUM

SAMPLE

Blood withdrawn during post-absorptive state. Anticoagulant—potassium oxalate; a fluoride-oxalate-thymol mixture (Reagent #1) is preferable if determinations cannot be made without undue delay. Prepare protein-free filtrate at once and keep in refrigerator until use.

TEST PROCEDURE

A. Macro

Special Apparatus

Ostwald-Folin pipettes accurately calibrated "to contain" 1 ml.

Folin sugar tubes, designed to minimize re-oxidation of the cuprous oxide by exposure to the air. It is important that these tubes adhere strictly to the specifications laid down by Folin. Satisfactory tubes, which are about 20 cm in length with an inside diameter of 17–18 mm, are constricted in the lower third of the tube for a distance of approximately 4 cm, the constriction ending about 2 cm from the bottom. The inside diameter of the constricted portion must not exceed 7–8 mm. The remainder of the tube below the constriction is blown into a spherical bulb. When 4 ml of fluid are placed in properly constructed tubes the level rises to not quite the mid-point of the constriction. Tubes must be accurately graduated at the 25 ml level. Summarizing, the essential points to be observed are: First, the inside diameter of the constricted portion must not exceed 8 mm; second, the bulb must be spherical in shape; and third, the 4 ml level must fall somewhere within the constriction. Tubes which do not meet these specifications should be rejected.

Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.

Reagents

1. **Fluoride-Oxalate-Thymol Mixture (Levinson-MacFate).** In a mortar place 10 gm powdered neutral sodium fluoride (NaF—Baker & Adamson, A.C.S.), 1 gm powdered thymol ($C_{10}H_{14}O$—Merck, Reagent) and 3 gm powdered neutral potassium oxalate ($K_2C_2O_4 \cdot H_2O$—J. T. Baker, A.C.S.); grind thoroughly until the components are intimately mixed. Fifteen milligrams are sufficient for the prevention of coagulation and the preservation of five milliliters of blood.
2. **Sulfuric Acid, Volumetric N/12.** Add 2.5 ml concentrated sulfuric acid (H_2SO_4—Grasselli, A.C.S.) to 1000 ml distilled water and mix well; titrate against standard 0.1 N alkali using phenolphthalein as indicator; adjust accordingly and re-titrate—20 ml of N/12 acid require 16.7 ml of 0.1 N alkali.
3. **Sodium Tungstate, 10% Aqueous.** Dissolve 10 gm sodium tungstate ($Na_2WO_4 \cdot 2H_2O$—Mallinckrodt, Folin Special) in distilled water and dilute to 100 ml. The solution must be neutral or slightly alkaline to phenolphthalein, not more than 0.4 ml being required to neutralize 10 ml 0.1 N hydrochloric acid. Preserve in resistant-glass bottle.
4. **Alkaline Copper Tartrate Reagent (Sahyun).** In a 1000 ml volumetric flask place 500 ml distilled water; add the following chemicals in the order named, shaking thoroughly after each addition to insure complete solution before the addition of the succeeding substance—17.75 gm anhydrous sodium sulfate (Na_2SO_4—Baker & Adamson, A.C.S.), 50 gm anhydrous sodium carbonate (Na_2CO_3—Baker & Adamson, A.C.S.), 7.5 gm tartaric acid ($H_2C_4H_4O_6$—Baker & Adamson, A.C.S.) and 4.5 gm crystalline copper sulfate ($CuSO_4 \cdot 5H_2O$—Baker & Adamson, A.C.S., Fine Crystals); dilute to 1000 ml with distilled water and mix; allow to stand for several days before use; filter if necessary—the solution must be clear. Preserve in ceresin-lined bottle. Avoid contamination by chlorine fumes.
5. **Phosphomolybdic Acid Reagent, Concentrated (Folin Improved).** In a 1000 ml Florence flask place 500 ml clear, supernatant, brominated sodium molybdate solution (a); add 225 ml 85%

phosphoric acid (H_3PO_4—Baker & Adamson, A.C.S.)—the solution becomes yellow due to liberated bromine; add 150 ml cool 25% sulfuric acid (b); aerate until free of bromine as evidenced by a colorless solution (usually in 10–15 minutes); transfer to a 1000 ml volumetric flask; add 75 ml glacial acetic acid (CH_3COOH—Baker & Adamson, A.C.S.); dilute to volume with distilled water and mix thoroughly. Stability indefinite.

- (a) Sodium molybdate, brominated; in a 1000 ml volumetric flask place 700 ml distilled water; add 300 gm sodium molybdate ($Na_2MoO_4 \cdot 2H_2O$—Mallinckrodt, AR) and shake until dissolved; add 2–3 drops bromine (Br—Baker & Adamson, A.C.S.); dilute to volume with distilled water and mix. On long standing a precipitate may form which does not affect the usefulness of the solution.
- (b) Sulfuric acid, 25% aqueous; dilute one volume concentrated sulfuric acid (H_2SO_4—Grasselli, A.C.S.) with three volumes distilled water; allow to cool before use.

6. **Phosphomolybdic Acid Reagent, Dilute.** In a 100 ml graduated mixing cylinder place 20 ml concentrated phosphomolybdic acid reagent (5) and dilute to volume with distilled water; mix thoroughly. Prepare fresh daily.

Technic

Note: For concentrations of dextrose in excess of 200 mg per 100 ml, use 1 ml protein-free filtrate and 1 ml distilled water in Step 7 and multiply the values obtained from the calibration table by 2.

1. In a 30 ml Erlenmeyer flask place
   8 ml N/12 sulfuric acid (2).
2. Add 1 ml sample from an Ostwald-Folin pipette thoroughly rinsing out the pipette by drawing in and expelling some of the mixture several times.
3. Mix the contents of the flask by gentle rotation until the fluid is uniformly black-brown.
4. Add 1 ml 10% sodium tungstate (3), drop by drop, while constantly, but gently, rotating the flask.
5. Shake the flask vigorously until the mixture is chocolate colored and foam-free.
6. Filter through a dry 9 cm Whatman #4 paper into a dry test tube.
7. In a Folin sugar tube (sample) place
   2 ml protein-free filtrate, placing the tip of the pipette in the bulb of the tube so that the fluid does not flow down the walls.
8. In another Folin sugar tube (reagent blank) place
   2 ml distilled water.
9. To each tube add 2 ml alkaline copper tartrate reagent (4)—the level of the fluid should now fall within the constricted portion of the tube.
10. Mix by lateral shaking and place the tubes in actively boiling water for 8 minutes.
11. Place in cold running water for 3 minutes.
12. Add 4 ml concentrated phosphomolybdic acid reagent (5), allowing the reagent to flow rapidly down the side of the tube.
13. Replace in boiling water for exactly 5 minutes.
14. Cool as before for 3 minutes.
15. Dilute to the 25 ml level with *dilute* phosphomolybdic acid reagent (6).
16. Mix thoroughly by inverting the tubes three times, allowing the bulbs to empty each time.
17. Allow to stand at room temperature for 2 minutes.
18. Measure the transmittancy of the sample against the reagent blank set at 100% transmission at a wave length of **500** $m\mu$ and obtain the concentration of dextrose from the calibration table.

CALIBRATION PROCEDURE

Special Apparatus

Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.

Folin sugar tubes; the 25 ml graduation must be accurately calibrated.

Reagents

4. **Alkaline Copper Tartrate Reagent (Sahyun).** Same as for Test Procedure.
5. **Phosphomolybdic Acid Reagent, Concentrated (Folin Improved).** Same as for Test Procedure.
6. **Phosphomolybdic Acid Reagent, Dilute.** Same as for Test Procedure.
7. **Benzoic Acid, 0.25% Aqueous.** In a 1500 ml beaker heat 1000 ml distilled water to boiling; add 2.5 gm benzoic acid ($C_7H_6O_2$—Baker & Adamson, Reagent) and stir until dissolved; allow to cool; adjust volume to 1000 ml with distilled water; filter.
8. **Dextrose Standards**
   - (a) Dextrose stock standard (1 ml contains 10 mg dextrose). Dry dextrose ($C_6H_{12}O_6$—National Bureau of Standards) over sulfuric acid in vacuo to constant weight; transfer 1 gm to a 100 ml volumetric flask; add 50 ml 0.25% benzoic acid (7); shake until dissolved; dilute to volume with same and mix. Stability indefinite.
   - (b) Dilute standard (1 ml contains 2 mg dextrose). In a 100 ml volumetric flask place 20 ml stock standard (8a) and dilute to volume with 0.25% benzoic acid (7); mix. Stable for approximately one month in refrigerator.
   - (c) Calibration standards. In a series of five accurately calibrated 100 ml volumetric flasks place respectively 0, 2.5, 5, 7.5 and 10 ml of the dilute standard (8b) and dilute to volume with distilled water; mix. These standards represent concentrations of dex-

trose equivalent to 0, 50, 100, 150 and 200 mg per 100 ml sample. Prepare just before use.

Technic

1. Transfer 2 ml of each calibration standard (8c) to a correspondingly marked Folin sugar tube.
2. Proceed exactly as outlined in the Test Procedure, Steps 9–17 inclusive.
3. Set the zero concentration standard at 100% transmission at a wave length of 500 mμ and record the transmittancy of the 50, 100, 150 and 200 mg per cent standards. The transmittancy of the 200 mg standard should be approximately 16%.
4. Plot the observed values on semi-log graph paper—the points fall on a straight line which passes through the origin (Calibration Curve D-10).
5. Prepare a calibration table from the T-C values obtained from the plotted curve.

Method D-10b

B. Micro

Special Apparatus

Byrd collecting and mixing pipettes. This pipette has two bulbs, the lower of which has a capacity of 0.1 ml from the tip to the first graduation; the total capacity from the tip to the upper graduation is 0.8 ml. The upper bulb contains a glass bead to aid in mixing. A mouthpiece and rubber tubing are attached.

Centrifuge tubes, 5 ml.

Ostwald-Folin pipettes accurately calibrated "to deliver" 0.5 ml.

Folin sugar tubes, Byrd modification—the 1 ml level must fall within the constriction of the tube; tubes must be accurately graduated at the 6.25 ml level.

Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.

Reagents

2. **Sulfuric Acid, Volumetric N/12.** Same as for Macro Procedure.
3. **Sodium Tungstate, 10% Aqueous.** Same as for Macro Procedure.
4. **Alkaline Copper Tartrate Reagent (Sahyun).** Same as for Macro Procedure.
5. **Phosphomolybdic Acid Reagent, Concentrated (Folin Improved).** Same as for Macro Procedure.
6. **Phosphomolybdic Acid Reagent, Dilute.** Same as for Macro Procedure.
9. **Potassium Oxalate, 10% Aqueous.** Dissolve 10 gm crystalline neutral potassium oxalate ($K_2C_2O_4 \cdot H_2O$—J. T. Baker, A.C.S.) in distilled water and dilute to 100 ml.

Technic

Note: For concentrations of dextrose in excess of 200 mg per 100 ml, dilute 1 volume of the protein-free filtrate with 1 volume of distilled water before proceeding with Step 10 and multiply the values obtained from the calibration table for the macro procedure by 2.

1. Into a dry Byrd pipette fitted with rubber tubing and mouthpiece draw 10% potassium oxalate (9) to slightly above the 0.1 ml graduation and expel—the thin film of oxalate remaining is sufficient to prevent coagulation of the blood.
2. Draw blood from a finger puncture to exactly the 0.1 ml graduation—wipe off any blood adhering to the outside of the pipette with clean tissue.
3. Insert the tip of the pipette into a 5 ml centrifuge tube so that the tip almost touches the bottom of the tube and expel the blood.
4. Draw N/12 sulfuric acid (2) into the pipette to the 0.8 ml graduation and shake thoroughly.
5. Expel the acid as described above into the blood in the tip of the centrifuge tube.
6. Mix the diluted blood until hemolysis is complete by gently twirling the tube between the hands.
7. Add 0.1 ml 10% sodium tungstate (3) and mix as above.
8. Allow to stand for 5 minutes and centrifuge at 1500 rpm for 5 minutes.
9. By means of an Ostwald-Folin pipette fitted with rubber tubing and mouthpiece, carefully withdraw slightly more than 0.5 ml clear supernatant centrifugate.
10. Adjust volume to exactly 0.5 ml and expel the contents into the bottom of a Byrd sugar tube (sample).
11. In another Byrd sugar tube (reagent blank) place 0.5 ml distilled water.
12. To each tube add 0.5 ml alkaline copper tartrate reagent (4).
13. Mix by lateral shaking and place in actively boiling water for 8 minutes.
14. Place in cold running water for 3 minutes.
15. Add 1 ml concentrated phosphomolybdic acid reagent (5), allowing the reagent to flow rapidly down the side of the tube.
16. Replace in boiling water for exactly 5 minutes.
17. Cool as before for 3 minutes.
18. Dilute to the 6.25 ml level with *dilute* phosphomolybdic acid reagent (6).
19. Mix thoroughly by inverting the tubes three times, allowing the bulbs to empty each time.

20. Allow to stand for 2 minutes.
21. Measure the transmittancy of the sample against the reagent blank set at 100% transmission at a wave length of **500** mμ and obtain the concentration of dextrose directly from the calibration table for the macro procedure.

Method D-10c

DETERMINATION IN URINE

SAMPLE

Aliquot of a 24-hour specimen. Keep cool during collection.

TEST PROCEDURE

Special Apparatus

As listed under the Macro Test Procedure for blood.

Reagents

2. **Sulfuric Acid, Volumetric N/12.** Same as for blood.
3. **Sodium Tungstate, 10% Aqueous.** Same as for blood.
4. **Alkaline Copper Tartrate Reagent (Sahyun).** Same as for blood.
5. **Phosphomolybdic Acid Reagent, Concentrated (Folin Improved).** Same as for blood.
6. **Phosphomolybdic Acid Reagent, Dilute.** Same as for blood.
10. **Oxalic Acid, Volumetric 0.05 N.** In a 1000 ml volumetric flask place 3.1518 gm crystalline oxalic acid ($H_2C_2O_4 \cdot 2H_2O$—J. T. Baker, Special for Standardizing); add 800 ml distilled water and shake until dissolved; dilute to volume with distilled water.
11. **Lloyd's Reagent (Lilly).** Other suitable adsorbents may be used.

Technic

Note: Determine the approximate concentration of dextrose by means of Benedict's qualitative test and select the appropriate procedure from one of the following:

A. For concentrations of dextrose less than 1%—dilute 1 ml filtrate to 5 ml in Step 4; proceed as outlined and multiply the values obtained from the calibration table for blood by 10.

B. For concentrations of dextrose from 1–4%—proceed exactly as outlined and multiply the values obtained from the calibration table for blood by 20.

C. For concentrations of dextrose in excess of 4%—dilute 1 ml filtrate to 20 ml in Step 4; proceed as outlined and multiply the values obtained from the calibration table for blood by 40.

In the absence of protein, as determined by the sulfosalicylic acid qualitative test, Steps 5–10 inclusive may be omitted provided that the proper dilution is made in Step 4 (1 ml filtrate diluted to 50 ml for concentrations less than 1%; 1 ml to 100 ml for concentrations of 1–4%; and 1 ml to 200 ml for concentrations in excess of 4%).

1. In a 50 ml Erlenmeyer flask place
   10 ml thoroughly mixed urine
   10 ml 0.05 N oxalic acid (10) and mix.
2. Add 1.5 gm Lloyd's reagent (11), stopper and shake vigorously for 3 minutes.
3. Filter through a dry paper into a dry test tube.
4. Dilute 1 ml filtrate to 10 ml with distilled water—the dilution in this step is made according to one of the selected procedures.
5. In a 30 ml Erlenmeyer flask place
   8 ml N/12 sulfuric acid (2).
6. Add 1 ml diluted urine from an Ostwald-Folin pipette, thoroughly rinsing out the pipette by drawing in and expelling some of the mixture several times.
7. Mix the contents of the flask by gentle rotation.
8. Add 1 ml 10% sodium tungstate (3), drop by drop while constantly, but gently, rotating the flask.
9. Shake the flask vigorously until the mixture is foam-free.
10. Filter through a dry 9 cm Whatman #4 paper into a dry test tube.
11. Proceed exactly as outlined in the Macro Test Procedure for blood, Steps 7–17 inclusive.
12. Measure the transmittancy of the sample against the reagent blank set at 100% transmission at a wave length of **500** mμ and multiply the value from the calibration table for blood by the appropriate factor (20 in the procedure outlined) for the selected procedure to obtain the concentration of dextrose in mg per 100 ml urine.

Calculation

To obtain the concentration of dextrose in per cent—

$$\frac{\text{Dextrose in mg per 100 ml}}{1000} = \text{Dextrose in \%}$$

$$\frac{\text{Volume of 24-hour specimen}}{100} \times \text{Dextrose in mg per 100 ml} = \text{Mg dextrose excreted in 24 hours}$$

Method D-10d

DETERMINATION IN CEREBROSPINAL FLUID

SAMPLE

Cerebrospinal fluid withdrawn during post-absorptive state. Determinations should be made without undue delay or the protein-free filtrate prepared and kept in the refrigerator until use. In the event of unavoidable delay, a suitable preservative may be added to the spinal fluid which is then kept in the refrigerator until the analysis can be carried out. The admixture of whole or hemolyzed blood or the presence of partially decomposed blood vitiates the results. For the proper interpretation of results the determination should also be carried out on a sample of blood withdrawn at the same time.

TEST PROCEDURE

Special Apparatus

Ostwald-Folin pipettes accurately calibrated "to contain" 2 ml.

Other apparatus as listed under the Macro Test Procedure for blood.

Reagents

2. **Sulfuric Acid, Volumetric N/12.** Same as for blood.
3. **Sodium Tungstate, 10% Aqueous.** Same as for blood.
4. **Alkaline Copper Tartrate Reagent (Sahyun).** Same as for blood.
5. **Phosphomolybdic Acid Reagent, Concentrated (Folin Improved).** Same as for blood.
6. **Phosphomolybdic Acid Reagent, Dilute.** Same as for blood.

Technic

Note: For concentrations of dextrose in excess of 100 mg per 100 ml, use 1 ml sample and 1 ml distilled water in Step 1 and obtain the values directly from the calibration table for blood.

1. For fluids with normal protein content—
    In a 15 ml centrifuge tube place
        7.6 ml N/12 sulfuric acid (2)
        2 ml cerebrospinal fluid from an Ostwald-Folin pipette, thoroughly rinsing out the pipette by drawing in and expelling some of the mixture several times
        0.4 ml 10% sodium tungstate (3) and mix thoroughly

    or

    For fluids with increased protein content—
    In a 15 ml centrifuge tube place
        7.2 ml N/12 sulfuric acid (2)
        2 ml cerebrospinal fluid from an Ostwald-Folin pipette, thoroughly rinsing out the pipette by drawing in and expelling some of the mixture several times
        0.8 ml 10% sodium tungstate (3) and mix thoroughly.
2. Centrifuge at 1500 rpm for 5 minutes.
3. Proceed exactly as outlined in the Macro Test Procedure for blood, Steps 7–17 inclusive.
4. Measure the transmittancy of the sample against the reagent blank set at 100% transmission at a wave length of **500** mμ and divide the value from the calibration table for blood by 2 to obtain the concentration of dextrose in mg per 100 ml cerebrospinal fluid.

PROCEDURE FOR USE WITH LINEAR-SCALE PHOTOELECTRIC COLORIMETERS (SHEARD-SANFORD TYPE)

TEST PROCEDURE

Exactly as outlined; set the reagent blank at 100 and use a blue filter.

CALIBRATION PROCEDURE

Exactly as outlined—use a blue filter; plot the observed readings on semi-log graph paper—the points fall on a straight line passing through the origin.

PROCEDURE FOR USE WITH LOGARITHMIC-SCALE PHOTOELECTRIC COLORIMETERS (KLETT-SUMMERSON TYPE)

TEST PROCEDURE

Exactly as outlined; set the reagent blank at 0 and use a blue filter.

CALIBRATION PROCEDURE

Exactly as outlined—use a blue filter; plot the observed readings on linear graph paper—the points fall on a straight line passing through the origin.

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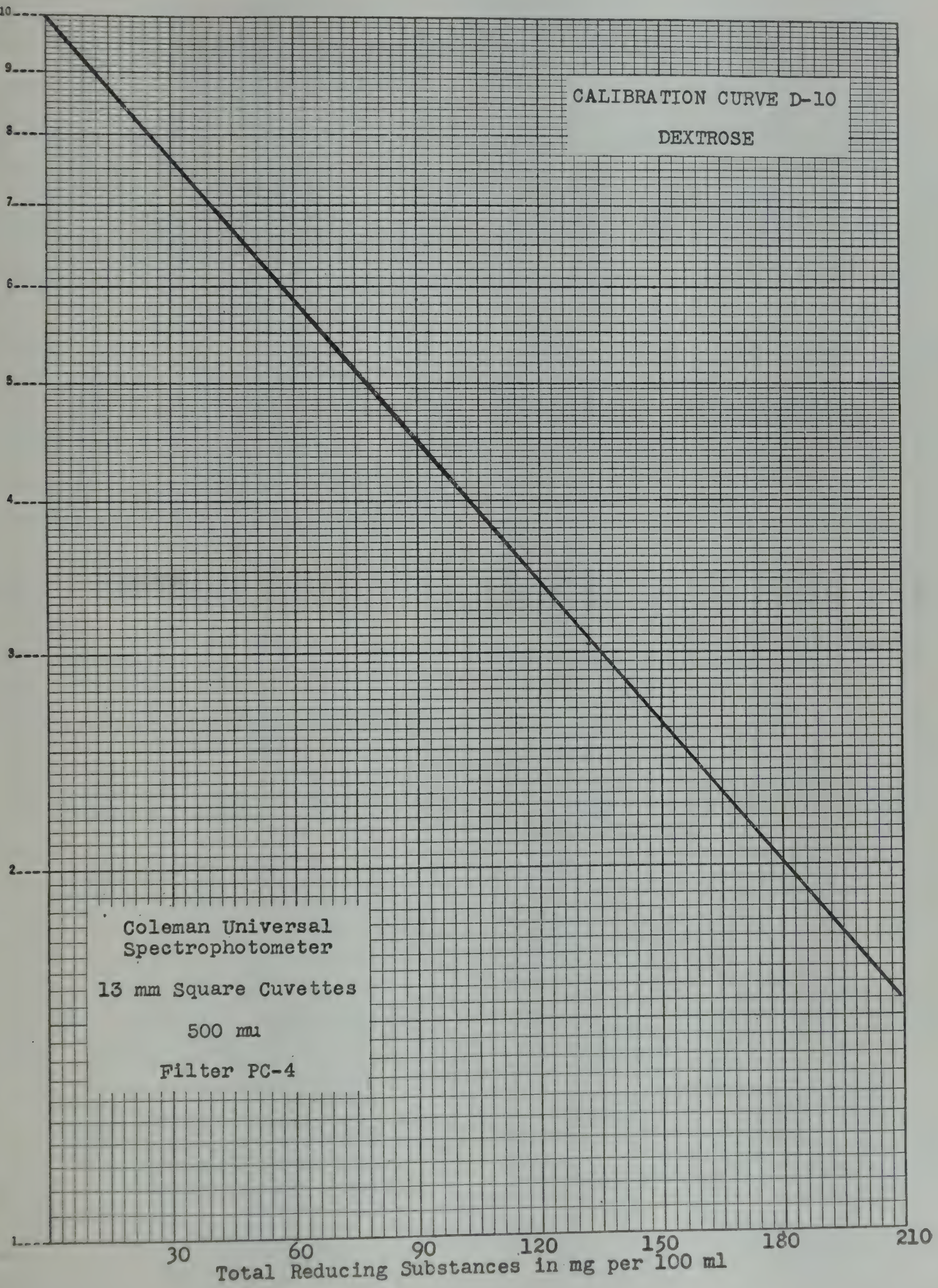
NOTES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DEXTROSE
Coleman Universal Spectrophotometer
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Filter PC-4
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4
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30
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150
180
210
Total Reducing Substances in mg per 100 ml

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70										
80										
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DEXTROSE, TRUE

A spectrophotometric adaptation based on the methods and modifications of Folin and Wu (1919), Nelson (1944) and Somogyi (1945)

PRINCIPLE

Dextrose, in a protein-free filtrate from which all other reducing substances have been removed by means of zinc sulfate-barium hydroxide precipitation, is heated with a copper reagent of high alkalinity. An amount of the copper proportional to the concentration of dextrose is reduced to cuprous oxide, and the latter, acting upon an arsenomolybdate reagent, reduces the molybdenum to a complex with an intense blue color of marked stability.

DETERMINATION IN WHOLE BLOOD, PLASMA OR SERUM

SAMPLE

Blood withdrawn during post-absorptive state. Anticoagulant—potassium oxalate; a fluoride-oxalate-thymol mixture (Reagent #1) is preferable if determinations cannot be made without undue delay. Prepare protein-free filtrate at once and keep in refrigerator until use.

TEST PROCEDURE

A. Macro

Special Apparatus

Ostwald-Folin pipettes accurately calibrated "to contain" 1 ml.

Folin sugar tubes, accurately graduated at the 15 ml level. For detailed specifications see the remarks in Method D-10a.

Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.

Reagents

1. **Fluoride-Oxalate-Thymol Mixture (Levinson-MacFate).** In a mortar place 10 gm powdered neutral sodium fluoride (NaF—Baker & Adamson, A.C.S.), 1 gm powdered thymol ($C_{10}H_{14}O$—Merck, Reagent) and 3 gm powdered neutral potassium oxalate ($K_2C_2O_4 \cdot H_2O$—J. T. Baker, A.C.S.); grind thoroughly until the components are intimately mixed. Fifteen milligrams are sufficient for the prevention of coagulation and the preservation of five milliliters of blood.
2. **Barium Hydroxide, Volumetric 0.3 N.** Place 600 ml distilled water in a 1000 ml Florence flask and boil for 5 minutes; add 45 gm crystalline barium hydroxide ($Ba(OH)_2 \cdot 8H_2O$—J. T. Baker, A.C.S.) and continue boiling until completely dissolved; immediately fit the flask with a 2-hole rubber stopper carrying syphon tubes, to each of which is attached a soda lime tube; allow to stand until cold—the strength is approximately 0.3 N; syphon off a small amount and titrate against 0.1 N oxalic acid using phenolphthalein as indicator; calculate the amount necessary to be diluted to 20 ml (or the quantity used in one day) with freshly boiled CO_2-free distilled water to make the strength exactly 0.3 N. Every day, or just before use, make up the volume required—do not dilute the entire stock solution at one time. The solution must be clear and contain no suspended barium carbonate.
3. **Zinc Sulfate, 5% Aqueous.** Dissolve 50 gm crystalline zinc sulfate ($ZnSO_4 \cdot 7H_2O$—J. T. Baker, A.C.S.) in distilled water and dilute to 1000 ml. Titrate against the standardized 0.3 N barium hydroxide (2) with phenolphthalein as follows: place exactly 10 ml of the zinc sulfate in a titration flask and add approximately 100 ml freshly boiled distilled water; add the barium hydroxide, dropwise and with constant agitation, until a pink color persists for at least one minute—10 ml of the sulfate must require 10 ml of the hydroxide—if not, correct accordingly.
4. **Copper-Phosphate-Tartrate Reagent, High Alkalinity (Somogyi).** In a 1500 ml beaker place 700 ml distilled water; add 28 gm anhydrous dibasic sodium phosphate (Na_2HPO_4—Mallinckrodt, AR) and stir until dissolved; add 40 gm crystalline sodium potassium tartrate ($NaKC_4H_4O_6 \cdot 4H_2O$—Baker & Adamson, A.C.S.) and stir until dissolved; add 100 ml volumetric 1 N sodium hydroxide (a) and mix; add 80 ml 10% copper sulfate (b) with constant stirring; add 180 gm anhydrous sodium sulfate (Na_2SO_4—Baker & Adamson, Reagent Special); stir until solution is complete; transfer to a 1000 ml mixing cylinder and dilute to volume with distilled water; allow to stand for 2 days until any sediment has settled; filter through Whatman #4 paper. Preserve in resistant-glass bottle. This reagent is stable indefinitely and shows no deposition of cuprous oxide.
   - (a) Sodium hydroxide, volumetric 1 N; prepare by diluting slightly more than the required amount of concentrated carbonate-free sodium hydroxide solution (see Appendix) and standardize against 1 N acid using phenolphthalein as indicator. Preserve in ceresin-lined bottle.
   - (b) Copper sulfate, 10% aqueous; dissolve 10 gm finely crystalline copper sulfate ($CuSO_4 \cdot 5H_2O$—Baker & Adamson, A.C.S., Fine Crystals) in distilled water; dilute to 100 ml and filter.
5. **Arsenomolybdate Color Reagent (Nelson).** In a 1000 ml Florence flask place 25 gm ammonium

molybdate ($(NH_4)_6Mo_7O_{24} \cdot 4H_2O$—Mallinckrodt, AR) and dissolve in 450 ml distilled water; add 21 ml concentrated sulfuric acid (H_2SO_4—Grasselli, A.C.S.) and mix; dissolve 3 gm crystalline sodium acid arsenate ($Na_2HAsO_4 \cdot 7H_2O$—Baker & Adamson, Reagent) in 25 ml distilled water and add to the contents of the flask; mix and place in incubator at 37° C. for 48 hours. Preserve in brown glass-stoppered bottle.

Technic

Note: For concentrations of dextrose in excess of 200 mg per 100 ml, use 1 ml protein-free filtrate and 1 ml distilled water in Step 6 and multiply the values obtained from the calibration table by 2.

1. In a 30 ml Erlenmeyer flask place
   15 ml distilled water.
2. Add 1 ml sample from an Ostwald-Folin pipette, thoroughly rinsing out the pipette by drawing in and expelling some of the mixture several times.
3. Add 2 ml 0.3 N barium hydroxide (2) and mix by gently agitating the tube until the mixture becomes uniformly brown in color.
4. Add 2 ml 5% zinc sulfate (3) and shake vigorously.
5. Centrifuge at 1800 rpm for 5 minutes (or filter through a dry Whatman #40 paper into a dry test tube).
6. In a Folin sugar tube (sample) place
   2 ml centrifugate (or filtrate), placing the tip of the pipette near the bottom of the tube so that the fluid does not flow down the walls.
7. In another Folin sugar tube (reagent blank) place
   2 ml distilled water.
8. To each tube add 2 ml copper-phosphate-tartrate reagent (4) and mix by lateral shaking.
9. Place the tubes in boiling water for 10 minutes.
10. Cool under running tap water for 3 minutes.
11. Add 2 ml arsenomolybdate color reagent (5), allowing the reagent to flow rapidly down the side of the tube and mix by vigorous lateral shaking.
12. Allow to stand at room temperature for 2 minutes.
13. Dilute to 15 ml with distilled water and mix by inverting the tubes three times, allowing the bulbs to empty each time.
14. Measure the transmittancy of the sample against the reagent blank set at 100% transmission at a wave length of 500 mμ and obtain the concentration of true dextrose from the calibration table.

CALIBRATION PROCEDURE

Special Apparatus

Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.
Folin sugar tubes, accurately graduated at the 15 ml level.

Reagents

4. **Copper-Phosphate-Tartrate Reagent, High Alkalinity (Somogyi).** Same as for Test Procedure.
5. **Arsenomolybdate Color Reagent (Nelson).** Same as for Test Procedure.
6. **Benzoic Acid, 0.25% Aqueous.** In a 1500 ml beaker heat 1000 ml distilled water to boiling; add 2.5 gm benzoic acid ($C_7H_6O_2$—Baker & Adamson, Reagent) and stir until dissolved; allow to cool; adjust volume to 1000 ml with distilled water and filter.
7. **Dextrose Standards**
   (a) Dextrose stock standard (1 ml contains 10 mg dextrose). Dry dextrose ($C_6H_{12}O_6$—National Bureau of Standards) over sulfuric acid in vacuo to constant weight; transfer 1 gm to a 100 ml volumetric flask; add 50 ml 0.25% benzoic acid (6); shake until dissolved; dilute to volume with same and mix. Stability indefinite.
   (b) Dilute standard (1 ml contains 1 mg dextrose). In a 100 ml volumetric flask place 10 ml stock standard (7a) and dilute to volume with 0.25% benzoic acid (6); mix. Stable for approximately one month in refrigerator.
   (c) Calibration standards. In a series of five accurately calibrated 100 ml volumetric flasks place respectively 0, 2.5, 5, 7.5 and 10 ml of the dilute standard (7b) and dilute to volume with distilled water; mix. These standards represent concentrations of dextrose equivalent to 0, 50, 100, 150 and 200 mg per 100 ml sample. Prepare just before use.

Technic

1. Transfer 2 ml of each calibration standard (7c) to a correspondingly marked Folin sugar tube.
2. Proceed exactly as outlined in the Test Procedure, Steps 8–13 inclusive.
3. Set the zero concentration standard at 100% transmission at a wave length of 500 mμ and record the transmittancy of the 50, 100, 150 and 200 mg per cent standards. The transmittancy of the 200 mg standard should be approximately 25.5%.
4. Plot the observed values on semi-log graph paper —the points fall on a straight line which passes through the origin (Calibration Curve D-12A).
5. Prepare a calibration table from the T-C values obtained from the plotted curve.

Method D-12b

B. Micro

Special Apparatus

Micro pipettes accurately calibrated "to contain" 0.1 ml.

Folin sugar tubes, accurately graduated at the 25 ml level. For detailed specifications see the remarks in Method D-10a.

Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.

Reagents

2. **Barium Hydroxide, Volumetric 0.3 N.** Same as for Macro Procedure.
3. **Zinc Sulfate, 5% Aqueous.** Same as for Macro Procedure.
4. **Copper-Phosphate-Tartrate Reagent, High Alkalinity (Somogyi).** Same as for Macro Procedure.
5. **Arsenomolybdate Color Reagent (Nelson).** Same as for Macro Procedure.

Technic

Note: For concentrations of dextrose in excess of 200 mg per 100 ml, dilute 1 volume protein-free centrifugate with 1 volume distilled water before proceeding with Step 6 and multiply the values obtained from the calibration table by 2.

1. In a 15 ml centrifuge tube place
   3.5 ml distilled water.
2. Add 0.1 ml sample from a micro pipette, thoroughly rinsing out the pipette by drawing in and expelling some of the mixture several times.
3. Add 0.2 ml 0.3 N barium hydroxide (2) and mix by gently tapping the tube.
4. Add 0.2 ml 5% zinc sulfate (3) and mix by gently tapping the tube.
5. Centrifuge at 1800 rpm for 5 minutes.
6. Transfer 2 ml clear supernatant centrifugate to a Folin sugar tube (sample) by means of a 2 ml volumetric pipette equipped with a rubber aspirating bulb.
7. In another Folin sugar tube (reagent blank) place
   2 ml distilled water.
8. To each tube add 1 ml copper-phosphate-tartrate reagent (4) and mix.
9. Add 1 ml distilled water and mix by a rotatory motion.
10. Place the tubes in boiling water for 20 minutes.
11. Cool under running tap water for 3 minutes.
12. Add 1 ml arsenomolybdate color reagent (5) allowing the reagent to flow rapidly down the side of the tube and mix by lateral shaking.
13. Allow to stand at room temperature for 2 minutes.
14. Dilute to 25 ml with distilled water and mix by inverting the tubes three times, allowing the bulbs to empty each time.
15. Measure the transmittancy of the sample against the reagent blank set at 100% transmission at a wave length of **660** $m\mu$ and obtain the concentration of true dextrose from the calibration table.

CALIBRATION PROCEDURE

Special Apparatus

Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.

Folin sugar tubes, accurately graduated at the 25 ml level.

Reagents

4. **Copper-Phosphate-Tartrate Reagent, High Alkalinity (Somogyi).** Same as for Test Procedure.
5. **Arsenomolybdate Color Reagent (Nelson).** Same as for Test Procedure.
6. **Benzoic Acid, 0.25% Aqueous.** Same as for Macro Calibration Procedure.
8. **Dextrose Standards**
   - (a) Dextrose stock standard (1 ml contains 10 mg dextrose). This is the same as Reagent #7a under the Macro Calibration Procedure.
   - (b) Dilute standard (1 ml contains 1 mg dextrose). This is the same as Reagent #7b under the Macro Calibration Procedure.
   - (c) Calibration standards. In a series of five accurately calibrated 100 ml volumetric flasks place respectively 0, 2, 3, 4 and 5 ml of the dilute standard (8b) and dilute to volume with distilled water; mix. These standards represent concentrations of dextrose equivalent to 0, 80, 120, 160 and 200 mg per 100 ml sample. Prepare just before use.

Technic

1. Transfer 2 ml of each calibration standard (8c) to a correspondingly marked Folin sugar tube.
2. Proceed exactly as outlined in the Test Procedure, Steps 8–14 inclusive.
3. Set the zero concentration standard at 100% transmission at a wave length of **660** $m\mu$ and record the transmittancy of the 80, 120, 160 and 200 mg per cent standards. The transmittancy of the 200 mg standard should be approximately 29%.
4. Plot the observed values on semi-log graph paper—the points fall on a straight line which passes through the origin (Calibration Curve D-12B).
5. Prepare a calibration table from the T-C values obtained from the plotted curve.

PROCEDURE FOR USE WITH LINEAR-SCALE PHOTOELECTRIC COLORIMETERS (SHEARD-SANFORD TYPE)

Macro

TEST PROCEDURE

Exactly as outlined; set the reagent blank at 100 and use a blue filter.

CALIBRATION PROCEDURE

Exactly as outlined—use a blue filter; plot the observed readings on semi-log graph paper—the points fall on a straight line passing through the origin.

Micro

TEST PROCEDURE

Exactly as outlined; set the reagent blank at 100 and use a red filter.

CALIBRATION PROCEDURE

Exactly as outlined—use a red filter; plot the observed readings on semi-log graph paper—the points fall on a straight line passing through the origin.

PROCEDURE FOR USE WITH LOGARITHMIC-SCALE PHOTOELECTRIC COLORIMETERS (KLETT-SUMMERSON TYPE)

Macro

TEST PROCEDURE

Exactly as outlined; set the reagent blank at 0 and use a blue filter.

CALILRATION PROCEDURE

Exactly as outlined—use a blue filter; plot the observed readings on linear graph paper—the points fall on a straight line passing through the origin.

Micro

TEST PROCEDURE

Exactly as outlined; set the reagent blank at 0 and use a red filter.

CALIBRATION PROCEDURE

Exactly as outlined—use a red filter; plot the observed readings on linear graph paper—the points fall on a straight line passing through the origin.

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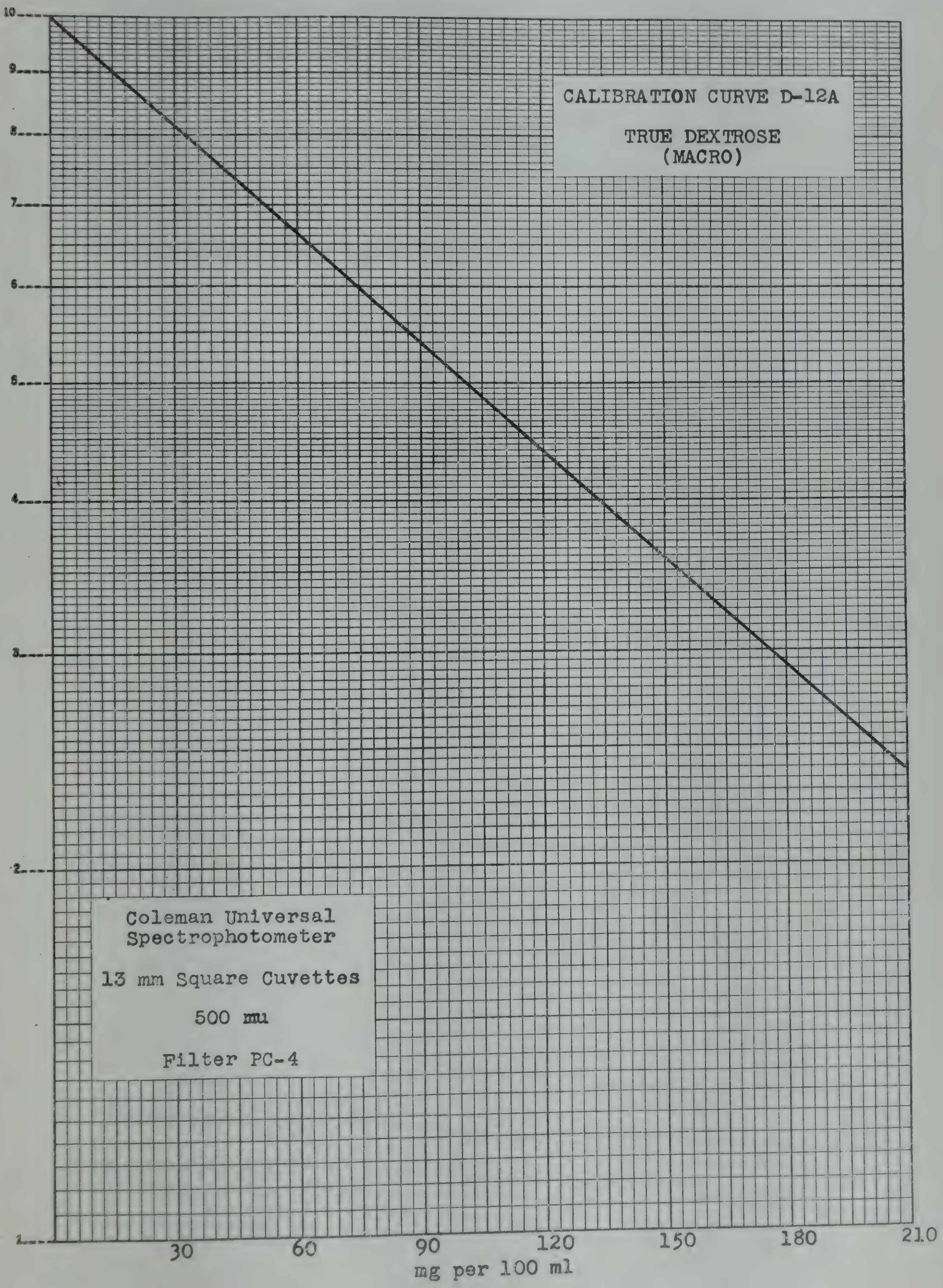
NOTES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mg per 100 ml

Meter Reading	0	1	2	3	4	5	6	7	8	9
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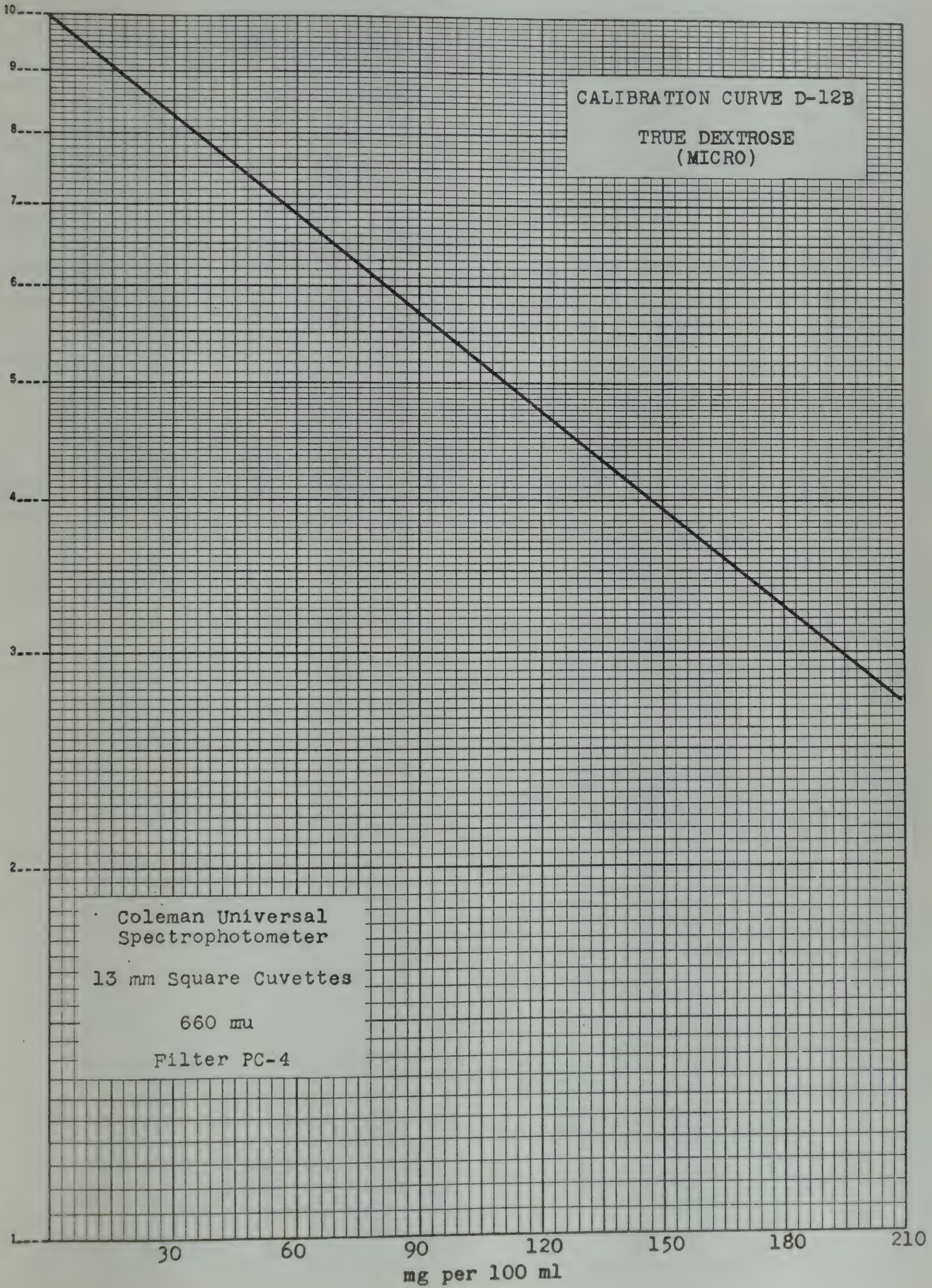
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mg per 100 ml

Meter Reading	0	1	2	3	4	5	6	7	8	9
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60										
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DEXTROSE

A spectrophotometric adaptation based on the methods and modifications of Folin and Malmros (1929), Jeghers and Myers (1930), Fujita and Iwatake (1931), Jourdonais (1938), Klendshoj and Hubbard (1940), Horvath and Knehr (1941) and Kingsley and Reinhold (1949)

PRINCIPLE

Blood is deproteinized by means of acid cadmium sulfate and sodium hydroxide yielding a filtrate containing true dextrose which is oxidized by alkaline potassium ferricyanide. The ferrocyanide produced is then converted to the Prussian blue ferric ferrocyanide.

DETERMINATION IN WHOLE BLOOD, PLASMA OR SERUM

SAMPLE

Blood withdrawn during post-absorptive state. Prepare protein-free centrifugate at once and keep in refrigerator until use. Refrigerated filtrates show no demonstrable loss of dextrose for several days.

TEST PROCEDURE

TRUE DEXTROSE

A. Macro

Special Apparatus

Ostwald-Folin pipettes accurately calibrated "to contain" 1 ml.

Graduated test tubes—25 ml in 0.5 ml; the 20 ml graduation must be accurately calibrated.

Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.

Reagents

1. **Acid Cadmium Sulfate Solution, Concentrated (Fujita-Iwatake).** In a 1000 ml volumetric flask place 13 gm crystalline cadmium sulfate ($3CdSO_4 \cdot 8H_2O$—Baker & Adamson, A.C.S.) or 10.59 gm anhydrous cadmium sulfate ($CdSO_4$—J. T. Baker, A.C.S.) and 63.5 ml 1 N sulfuric acid (a); add approximately 900 ml distilled water and shake until dissolved; dilute to volume with distilled water and filter. Note: If crystals of cadmium sulfate are used they must be clear and show no efflorescence.
   (a) Sulfuric acid, volumetric 1 N; slowly add 30 ml concentrated sulfuric acid (H_2SO_4—Grasselli, A.C.S.) to 1000 ml distilled water and mix; allow to cool; standardize against 1 N alkali using phenolphthalein as indicator.
2. **Sodium Hydroxide, Volumetric 1.1 N.** Prepare by diluting slightly more than the required amount of concentrated carbonate-free sodium hydroxide solution (see Appendix) and titrate against standard 1 N acid using phenolphthalein as indicator; adjust accordingly and re-titrate—20 ml of 1 N acid require 18.2 ml of 1.1 N alkali. Preserve in ceresin-lined bottle.
3. **Alkaline Potassium Ferricyanide Reagent (Kingsley-Reinhold).** In a 1000 ml volumetric flask place 1.84 gm anhydrous sodium carbonate (Na_2CO_3—Baker & Adamson, A.C.S.) and 0.8 gm recrystallized ferrocyanide-free potassium ferricyanide ($K_3Fe(CN)_6$—Reagent Chemicals, Special); add approximately 900 ml distilled water and shake until dissolved; dilute to volume and filter. Preserve in a brown glass bottle away from direct light. Discard when the reagent blank shows an appreciable increase.
4. **Ferric Iron Reagent (Kingsley-Reinhold).** In a 1000 ml volumetric flask place 2 gm crystalline ferric ammonium sulfate ($Fe_2(SO_4)_3(NH_4)_2SO_4 \cdot 24H_2O$—Baker & Adamson, A.C.S.); add approximately 900 ml distilled water and shake until dissolved; add 55 ml 85% phosphoric acid (H_3PO_4—Baker & Adamson, A.C.S.) and mix; dilute to volume with distilled water; mix and filter.

Technic

Note: For concentrations of dextrose in excess of 300 mg per 100 ml, dilute 1 ml of the protein-free centrifugate (or filtrate) with 1 ml distilled water before proceeding with Step 6 and multiply the values obtained from the calibration table by 2.

1. In a 25 ml Erlenmeyer flask place
   8 ml concentrated acid cadmium sulfate solution (1)
   1 ml sample from an Ostwald-Folin pipette, thoroughly rinsing out the pipette by drawing in and expelling some of the mixture several times.
2. Mix thoroughly by a rotatory motion and allow to stand for 1 minute.
3. Add 1 ml 1.1 N sodium hydroxide (2) and shake vigorously until foam-free.
4. Allow to stand for 15 minutes.
5. Centrifuge at 3000 rpm for 5 minutes (or filter through a Whatman #40 paper)—the protein-free fluid must be crystal clear.
6. In a 25 ml graduated test tube (sample) place
   0.5 ml centrifugate (or filtrate)
   5 ml alkaline potassium ferricyanide reagent (3) and mix by whirling.

7. In another 25 ml graduated test tube (reagent blank) place
   0.5 ml distilled water
   5 ml alkaline potassium ferricyanide reagent (3) and mix.
8. Place tubes in actively boiling water for 10 minutes.
9. Cool tubes in water bath at 40° C. for 3 minutes.
10. To each tube add 5 ml ferric iron reagent (4) and mix.
11. Replace tubes in water bath at 40° C. and allow to remain for 5 minutes.
12. Dilute to 20 ml with distilled water and mix by inversion.
13. Immediately measure the transmittancy of the sample against the reagent blank set at 100% transmission at a wave length of **540** mμ and obtain the concentration of dextrose from the calibration table.

CALIBRATION PROCEDURE

Special Apparatus

Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.

Graduated test tubes—25 ml in 0.5 ml; the 20 ml graduation must be accurately calibrated.

Reagents

3. **Alkaline Potassium Ferricyanide Reagent (Kingsley-Reinhold).** Same as for Test Procedure.
4. **Ferric Iron Reagent (Kingsley-Reinhold).** Same as for Test Procedure.
5. **Benzoic Acid, 0.25% Aqueous.** In a 1500 ml beaker heat 1000 ml distilled water to boiling; add 2.5 gm benzoic acid ($C_7H_6O_2$—Baker & Adamson, Reagent) and stir until dissolved; allow to cool; adjust volume to 1000 ml with distilled water and filter.
6. **Dextrose Standards**
   (a) Dextrose stock standard (1 ml contains 10 mg dextrose). Dry dextrose ($C_6H_{12}O_6$—National Bureau of Standards) over sulfuric acid in vacuo to constant weight; transfer 1 gm to a 100 ml volumetric flask; add 50 ml 0.25% benzoic acid (5); shake until dissolved; dilute to volume with same and mix. Stability indefinite.
   (b) Dilute standard (1 ml contains 2 mg dextrose). In a 100 ml volumetric flask place 20 ml stock standard (6a) and dilute to volume with 0.25% benzoic acid (5); mix. Stability approximately one month in refrigerator.
   (c) Calibration standards. In a series of seven accurately calibrated 100 ml volumetric flasks place respectively 0, 2.5, 5.0, 7.5, 10, 12.5 and 15 ml of the dilute standard (6b) and dilute to volume with distilled water. These standards represent concentrations of dextrose equivalent to 0, 50, 100, 150, 200, 250 and 300 mg per 100 ml sample. Prepare just before use and proceed immediately as follows.

Technic

1. Transfer 0.5 ml of each calibration standard (6c) to a correspondingly marked 25 ml graduated test tube.
2. To each tube add 5 ml alkaline potassium ferricyanide reagent (4) and mix by whirling.
3. Proceed exactly as outlined in the Test Procedure, Steps 8–12 inclusive.
4. Immediately (completing all measurements within 20 minutes after dilution) set the zero concentration standard at 100% transmission at a wave length of **540** mμ and record the transmittancy of the 50, 100, 150, 200, 250 and 300 mg per cent standards. The transmittancy of the 300 mg standard should be approximately 18.5%.
5. Plot the observed values on semi-log graph paper—the points fall on a straight line which passes through the origin (Calibration Curve D-14A).
6. Prepare a calibration table from the T-C values obtained from the plotted curve.

Method D-14b

B. Micro

Special Apparatus

Centrifuge tubes—5 ml; the 1 ml graduation must be accurately calibrated.

Micro pipettes accurately calibrated "to contain" 20 cmm.

Graduated test tubes—10 ml in 0.1 ml; the 6 ml graduation must be accurately calibrated.

Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.

Reagents

3. **Alkaline Potassium Ferricyanide Reagent (Kingsley-Reinhold).** Same as for Macro Procedure.
4. **Ferric Iron Reagent (Kingsley-Reinhold).** Same as for Macro Procedure.
7. **Acid Cadmium Sulfate Solution, Dilute (Fujita-Iwatake).** Dilute 36 ml concentrated acid cadmium sulfate solution (1) to 100 ml with distilled water.

8. **Sodium Hydroxide, Volumetric 0.022 N.** Dilute 2 ml 1.1 N sodium hydroxide (2) to 100 ml with distilled water. Preserve in ceresin-lined bottle.

Technic

Note: For concentrations of dextrose in excess of 400 mg per 100 ml, dilute 1 ml of the protein-free centrifugate with 1 ml distilled water before proceeding with Step 7 and multiply the values obtained from the calibration table by 2.

1. In a 5 ml centrifuge tube place
   0.5 ml *dilute* acid cadmium sulfate solution (7)
   0.02 ml sample from a micro pipette, thoroughly rinsing out the pipette by drawing in and expelling some of the mixture several times.
2. Dilute to exactly 1 ml with dilute acid cadmium sulfate solution (7).
3. Mix by twirling the tube between the hands and allow to stand for 1 minute.
4. Add 1 ml 0.022 N sodium hydroxide (8) and shake vigorously until foam-free.
5. Allow to stand for 15 minutes.
6. Centrifuge at 3000 rpm for 5 minutes—the supernatant fluid must be crystal clear.
7. In a 10 ml graduated test tube (sample) place
   1 ml clear supernatant centrifugate
   1.5 ml alkaline potassium ferricyanide reagent (3) and mix by whirling.
8. In another 10 ml graduated test tube (reagent blank) place
   1 ml distilled water
   1.5 ml alkaline potassium ferricyanide reagent (3) and mix.
9. Place tubes in actively boiling water for 10 minutes.
10. Cool tubes in water bath at 40° C. for 3 minutes.
11. To each tube add 1.5 ml ferric iron reagent (4) and mix.
12. Replace tubes in water bath at 40° C. and allow to remain for 5 minutes.
13. Dilute to 6 ml with distilled water and mix by inversion.
14. Immediately measure the transmittancy of the sample against the reagent blank set at 100% transmission at a wave length of 540 mμ and obtain the concentration of dextrose from the calibration table.

CALIBRATION PROCEDURE

Special Apparatus

Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.

Graduated test tubes—10 ml in 0.1 ml; the 6 ml graduation must be accurately calibrated.

Reagents

3. **Alkaline Potassium Ferricyanide Reagent (Kingsley-Reinhold).** Same as for Test Procedure.
4. **Ferric Iron Reagent (Kingsley-Reinhold).** Same as for Test Procedure.
5. **Benzoic Acid, 0.25% Aqueous.** Same as for Macro Calibration Procedure.
9. **Dextrose Standards**
   (a) Dextrose stock standard (1 ml contains 10 mg dextrose). This is the same as Reagent #6a under the Macro Calibration Procedure.
   (b) Dilute standard (1 ml contains 1 mg dextrose). In a 100 ml volumetric flask place 10 ml stock standard (9a) and dilute to volume with 0.25% benzoic acid (5). Stability approximately one month in refrigerator.
   (c) Calibration standards. In a series of nine accurately calibrated 100 ml volumetric flasks place respectively 0, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5 and 4.0 ml of the dilute standard (9b) and dilute to volume with distilled water. These standards represent concentrations of dextrose equivalent to 0, 50, 100, 150, 200, 250, 300, 350 and 400 mg per 100 ml sample. Prepare just before use and proceed immediately as outlined.

Technic

1. Transfer 1 ml of each calibration standard (9c) to a correspondingly marked 10 ml graduated test tube.
2. To each tube add 1.5 ml alkaline potassium ferricyanide reagent (3) and mix by whirling.
3. Proceed exactly as outlined in the Test Procedure, Steps 9–13 inclusive.
4. Immediately (completing all measurements within 20 minutes after dilution) set the zero concentration standard at 100% transmission at a wave length of 540 mμ and record the transmittancy of the 50, 100, 150, 200, 250, 300, 350 and 400 mg per cent standards. The transmittancy of the 400 mg standard should be approximately 22%.
5. Plot the observed values on semi-log graph paper—the points fall on a straight line which passes through the origin (Calibration Curve D-14B).
6. Prepare a calibration table from the T-C values obtained from the plotted curve.

Note: The procedures as previously outlined determine the true dextrose content of the blood. If, for convenience or comparative purposes, the concentration of total reducing substances is desired, the methods may be modified as follows without necessitating a separate calibration procedure.

TOTAL REDUCING SUBSTANCES

Method D-14c

A. Macro

Special Apparatus

As listed under the Macro Test Procedure.

Reagents

3. **Alkaline Potassium Ferricyanide Reagent (Kingsley-Reinhold).** Same as for Macro Test Procedure.
4. **Ferric Iron Reagent (Kingsley-Reinhold).** Same as for Macro Test Procedure.
10. **Sulfuric Acid, Volumetric N/12.** Add 2.5 ml concentrated sulfuric acid (H_2SO_4—Grasselli, A.C.S.) to 1000 ml distilled water and mix well; titrate against standard 0.1 N alkali using phenolphthalein as indicator; adjust accordingly and retitrate—20 ml of N/12 acid require 16.7 ml of 0.1 N alkali.
11. **Sodium Tungstate, 10% Aqueous.** Dissolve 10 gm sodium tungstate ($Na_2WO_4 \cdot 2H_2O$—Mallinckrodt, Folin Special) in distilled water and dilute to 100 ml. The solution must be neutral or slightly alkaline to phenolphthalein, not more than 0.4 ml being required to neutralize 10 ml 0.1 N hydrochloric acid. Preserve in resistant-glass bottle.

Technic

1. In a 30 ml Erlenmeyer flask place
   8 ml N/12 sulfuric acid (10)
   1 ml sample from an Ostwald-Folin pipette, thoroughly rinsing out the pipette by drawing in and expelling some of the mixture several times.
2. Mix the contents of the flask by gentle rotation until the fluid is uniformly black-brown.
3. Add 1 ml 10% sodium tungstate (11), drop by drop while constantly, but gently, rotating the flask.
4. Shake the flask vigorously until the mixture is chocolate colored and foam-free.
5. Filter through a dry 9 cm Whatman #4 paper into a dry test tube.
6. Proceed exactly as outlined in the Macro Test Procedure, Steps 6–13 inclusive.

Method D-14d

B. Micro

Special Apparatus

As listed under the Micro Test Procedure.

Reagents

3. **Alkaline Potassium Ferricyanide Reagent (Kingsley-Reinhold).** Same as for Micro Test Procedure.
4. **Ferric Iron Reagent (Kingsley-Reinhold).** Same as for Micro Test Procedure.
12. **Tungstic Acid Solution (Sendroy).** In a 100 ml volumetric flask place 8 ml 10% sodium tungstate (11); add about 20 ml distilled water; while shaking the flask, slowly add 64 ml N/12 sulfuric acid (10); dilute to volume with distilled water and mix. The stability of this solution is limited—it should be prepared fresh every two weeks or oftener if a sediment appears. It must yield a water clear protein-free supernatant fluid and give no color with a blank analysis.

Technic

1. In a 5 ml centrifuge tube place
   0.5 ml tungstic acid solution (12)
   0.02 ml sample from a micro pipette, thoroughly rinsing out the pipette by drawing in and expelling some of the mixture several times.
2. Dilute to exactly 1 ml with tungstic acid solution (12).
3. Mix by twirling the tube between the hands and allow to stand for 10 minutes.
4. Centrifuge at 1800 rpm for 10 minutes.
5. Proceed exactly as outlined in the Micro Test Procedure, Steps 7–14 inclusive.

PROCEDURE FOR USE WITH LINEAR-SCALE PHOTOELECTRIC COLORIMETERS (SHEARD-SANFORD TYPE)

TEST PROCEDURES

Exactly as outlined; set the reagent blank at 100 and use a green filter.

CALIBRATION PROCEDURES

Exactly as outlined—use a green filter; plot the observed readings on semi-log graph paper—the points fall on a straight line passing through the origin.

PROCEDURE FOR USE WITH LOGARITHMIC-SCALE PHOTOELECTRIC COLORIMETERS (KLETT-SUMMERSON TYPE)

TEST PROCEDURES

Exactly as outlined; set the reagent blank at 0 and use a green filter.

CALIBRATION PROCEDURES

Exactly as outlined—use a green filter; plot the observed readings on linear graph paper—the points fall on a straight line passing through the origin.

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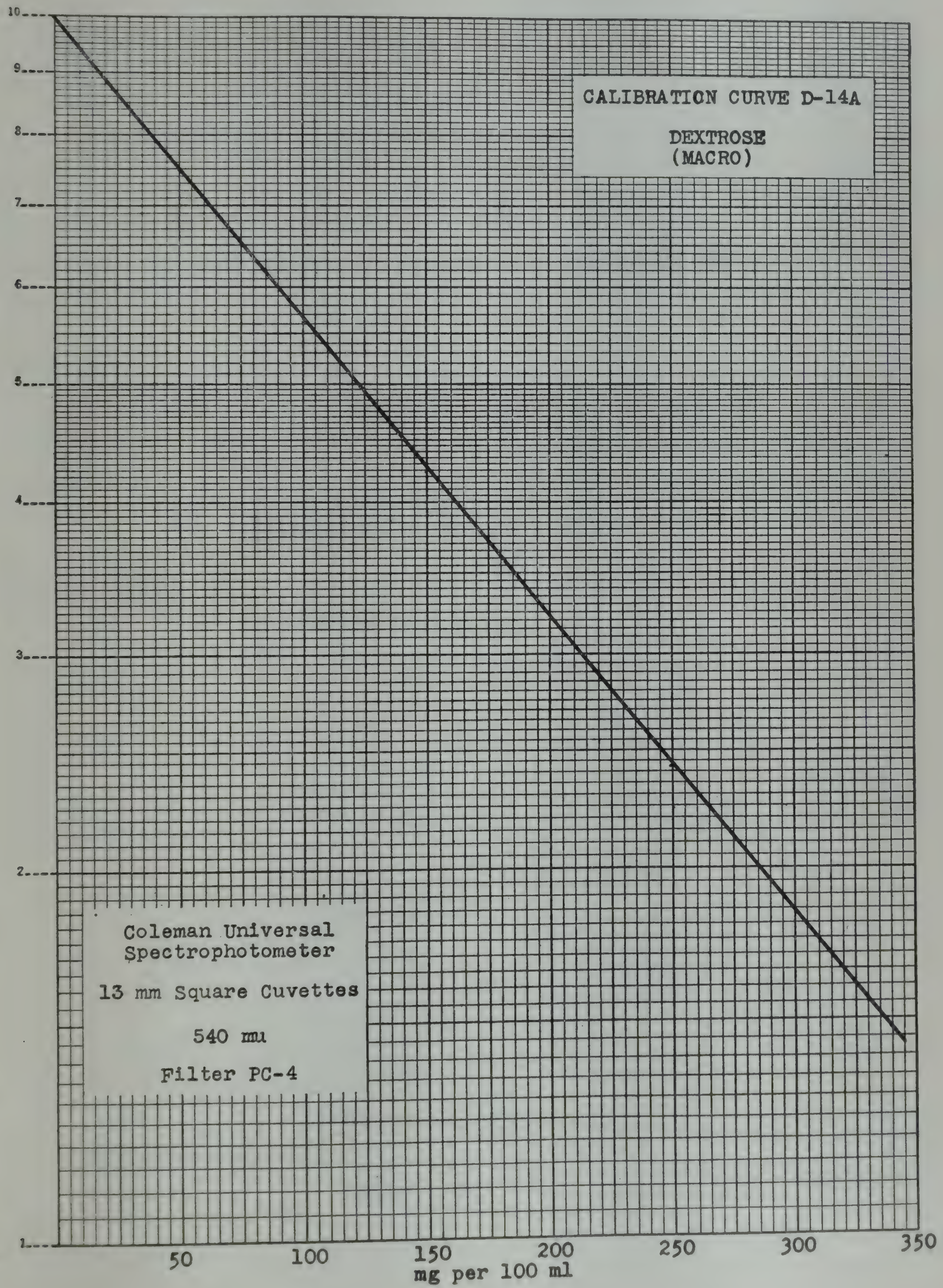
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6
5
4
3
2
1
50
100
150
200
250
300
350
mg per 100 ml

Meter Reading	0	1	2	3	4	5	6	7	8	9
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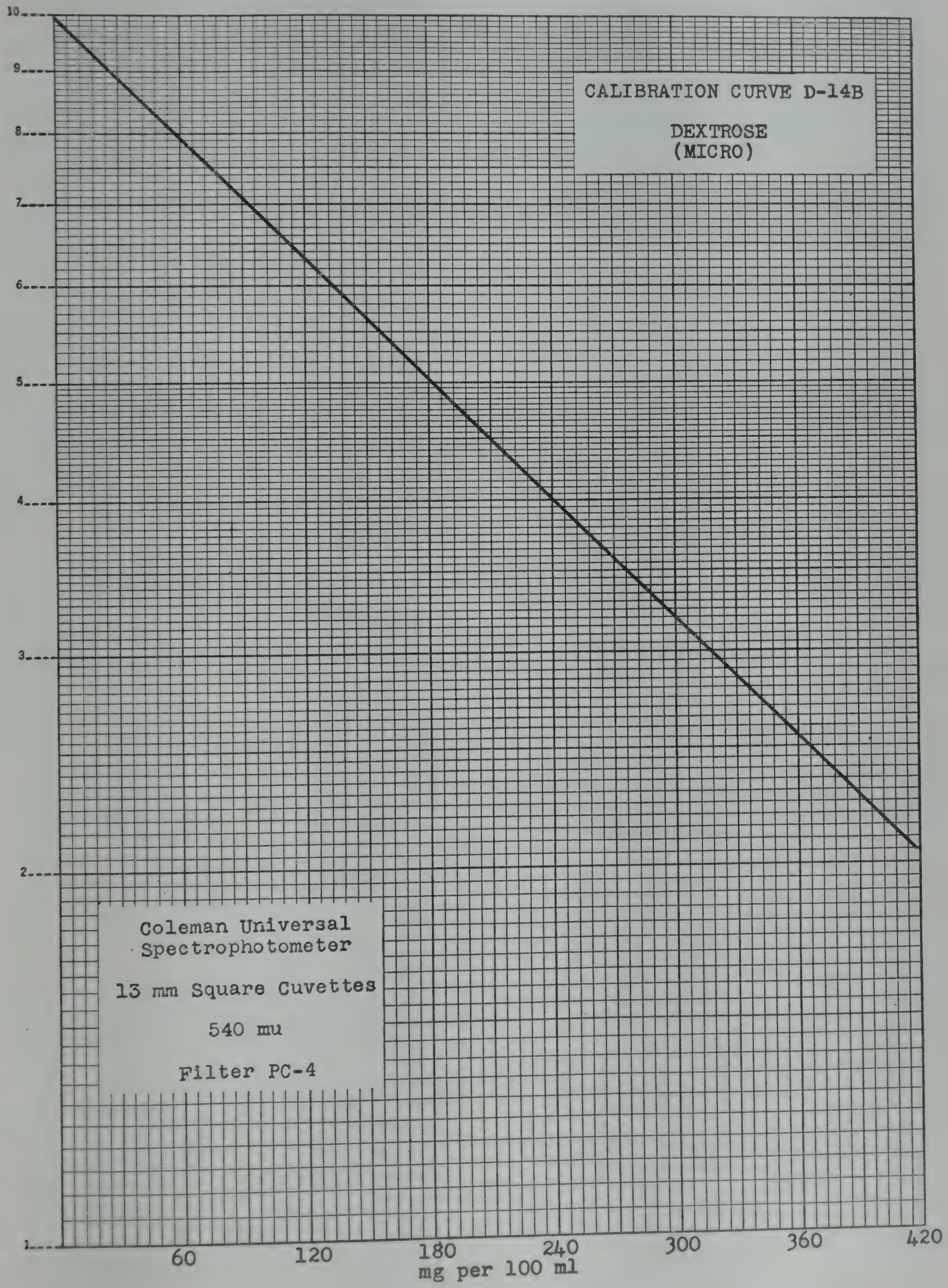
10										
20										
30										
40										
50										
60										
70										
80										
90										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(MICRO)
Coleman Universal
Spectrophotometer
13 mm Square Cuvettes
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Filter PC-4
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6
5
4
3
2
1
60
120
180
240
300
360
420
mg per 100 ml

Meter Reading	0	1	2	3	4	5	6	7	8	9
0										
10										
20										
30										
40										
50										
60										
70										
80										
90										

DEXTROSE

A spectrophotometric adaptation based on the method of Somogyi (1941)

PRINCIPLE

Dextrose, when heated with sodium carbonate, develops a color which ranges from pale yellow to dark amber dependent upon the concentration of sugar. Although this method is non-specific, in that other sugars react similarly, it is included here for routine clinical use.

DETERMINATION IN URINE

SAMPLE

Aliquot of a 24-hour specimen. Keep cool during collection. For samples which are clear and light in color proceed as outlined; for those abnormally dark in color or containing blood or bile pigments, add 3 gm Lloyd's Reagent (Reagent #2) to about 10 ml urine, shake gently for 2 minutes, filter and proceed with the filtrate as outlined.

TEST PROCEDURE

Special Apparatus

Graduated test tubes—25 ml in 0.5 ml; the 20 ml graduation must be accurately calibrated.
Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.

Reagents

1. **Sodium Carbonate, 10% Aqueous.** In a 1000 ml volumetric flask place about 500 ml distilled water, add 100 gm anhydrous sodium carbonate (Na_2CO_3—Baker & Adamson, A.C.S.) and shake until dissolved; dilute to volume and filter. Preserve in ceresin-lined bottle.
2. **Lloyd's Reagent (Lilly).** Other suitable adsorbents may be used.

Technic

Note: For concentrations of dextrose less than 0.4%, use 2 ml urine in both the sample and sample blank tubes and divide the values obtained from the calibration table by 2; for concentrations in excess of 5%, use 0.5 ml urine in both tubes and multiply the values by 2.

1. Filter a small aliquot of thoroughly mixed urine.
2. In a 25 ml graduated test tube (sample) place
   5 ml 10% sodium carbonate (1)
   1 ml filtered urine and mix.
3. In another 25 ml graduated test tube (sample blank) place
   5 ml distilled water
   1 ml filtered urine and mix.
4. Place both tubes in boiling water for 8 minutes.
5. Remove tubes from water bath and without cooling, dilute to 20 ml with distilled water and mix by inversion.
6. Immediately measure the transmittancy of the sample against the sample blank set at 100% transmission at a wave length of 500 mμ and obtain the concentration of dextrose from the calibration table.

Calculation

$$\frac{\text{Volume of 24-hour specimen}}{100} \times \text{Dextrose in \%} =$$
Gm dextrose excreted in 24 hours

CALIBRATION PROCEDURE

Special Apparatus

Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.
Graduated test tubes—25 ml in 0.5 ml; the 20 ml graduation must be accurately calibrated.

Reagents

1. **Sodium Carbonate, 10% Aqueous.** Same as for Test Procedure.
3. **Benzoic Acid, 0.25% Aqueous.** In a 1500 ml beaker heat 1000 ml distilled water to boiling; add 2.5 gm benzoic acid ($C_7H_6O_2$—Baker & Adamson, Reagent) and stir until dissolved; allow to cool; adjust volume to 1000 ml with distilled water and filter.
4. **Dextrose Standards**
   (a) Dextrose stock standard (1 ml contains 10 mg dextrose). Dry dextrose ($C_6H_{12}O_6$—National Bureau of Standards) over sulfuric acid in vacuo to constant weight; transfer 1 gm to a 100 ml volumetric flask; add 50 ml 0.25% benzoic acid (3); shake until dissolved; dilute to volume with same and mix. Stability indefinite.
   (b) Calibration standards. In a series of six accurately calibrated 25 ml graduated test tubes place respectively 0, 1, 2, 3, 4 and 5 ml stock standard (4a); add 5, 4, 3, 2, 1 and 0 ml distilled water respectively, bringing the volume in each tube to 5 ml. These standards represent concentrations of dextrose equivalent to 0, 1, 2, 3, 4 and 5%. Prepare just before use and proceed immediately as follows.

Technic

1. To each calibration standard (4b) add 5 ml 10% sodium carbonate (1) and mix.
2. Proceed exactly as outlined in the Test Procedure, Steps 4–5 inclusive.
3. Set the zero concentration standard at 100% transmission at a wave length of 500 mμ and record the transmittancy of the 1, 2, 3, 4 and 5% standards. The transmittancy of the 5% standard should be approximately 20%.
4. Plot the observed values on semi-log graph paper —the points fall on a smooth curve which passes through the concentration axis slightly to the right of the origin (Calibration Curve D-16).
5. Prepare a calibration table from the T-C values obtained from the plotted curve.

PROCEDURE FOR USE WITH LINEAR-SCALE PHOTOELECTRIC COLORIMETERS (SHEARD-SANFORD TYPE)

TEST PROCEDURE

Exactly as outlined; set the sample blank at 100 and use a green filter.

CALIBRATION PROCEDURE

Exactly as outlined—use a green filter; plot the observed readings on semi-log graph paper—the points fall on a smooth curve passing through the concentration axis slightly to the right of the origin.

PROCEDURE FOR USE WITH LOGARITHMIC-SCALE PHOTOELECTRIC COLORIMETERS (KLETT-SUMMERSON TYPE)

TEST PROCEDURE

Exactly as outlined; set the sample blank at 0 and use a green filter.

CALIBRATION PROCEDURE

Exactly as outlined—use a green filter; plot the observed readings on linear graph paper—the points fall on a straight line passing through the origin.

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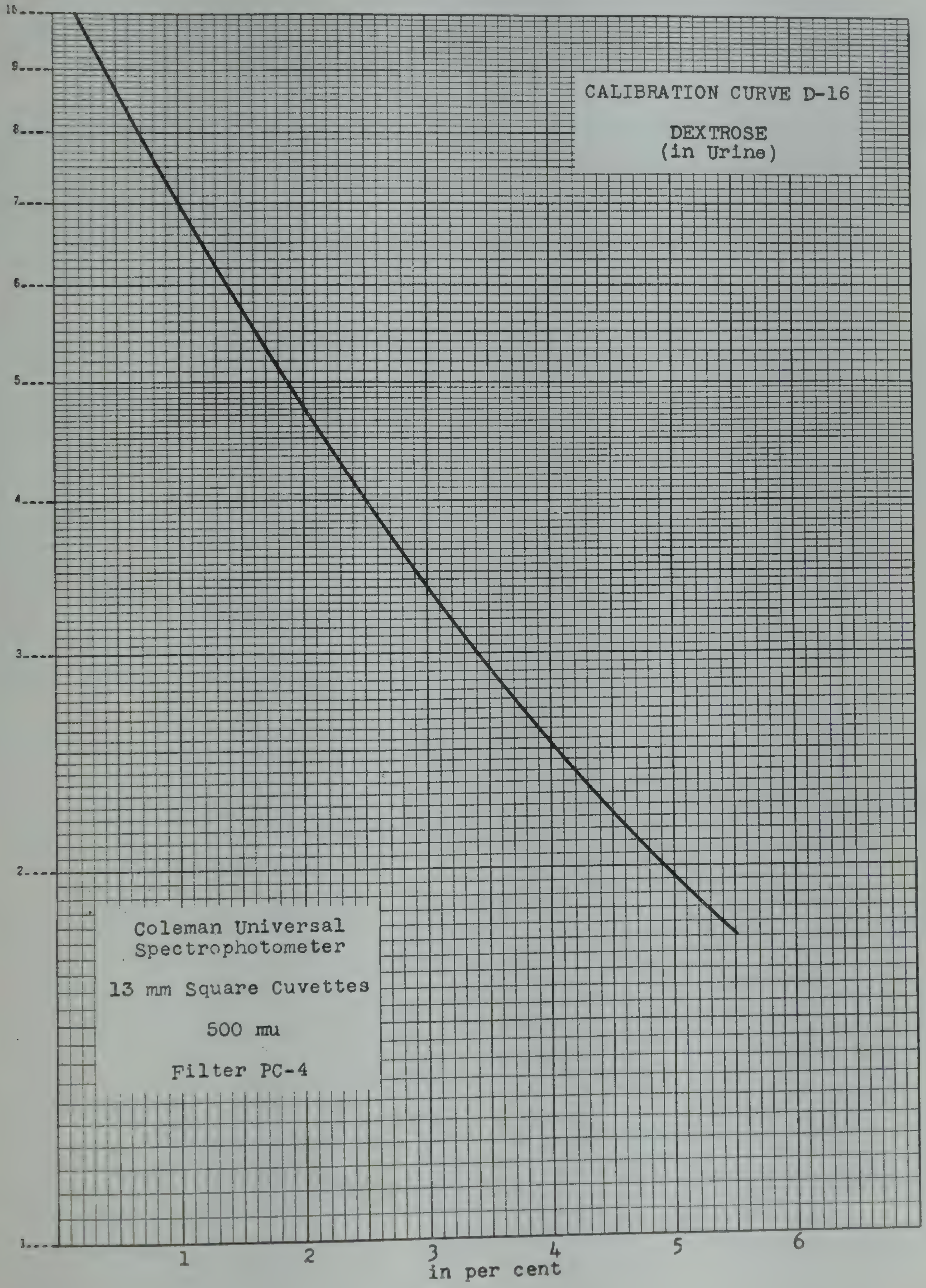
NOTES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(in Urine)
Coleman Universal
Spectrophotometer
13 mm Square Cuvettes
500 mu
Filter PC-4
10
9
8
7
6
5
4
3
2
1
1
2
3
4
5
6
in per cent

Meter Reading	0	1	2	3	4	5	6	7	8	9
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10										
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60										
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80										
90										

ETHYL ALCOHOL

A spectrophotometric adaptation based on the methods and modifications of Fleming and Stotz (1935) and Gibson and Blotner (1938)

PRINCIPLE

Alcohol, when distilled into an acid dichromate solution, reduces the dichromate (Nicloux reaction) with a resultant diminution of color. The unreduced dichromate is measured and thus, indirectly, the concentration of alcohol originally present is determined.

DETERMINATION IN WHOLE BLOOD

SAMPLE

Anticoagulant—potassium oxalate. Sterilization of instruments and skin must be made with a 1:1000 mercuric chloride solution instead of alcohol or any alcoholic germicide.

TEST PROCEDURE

Special Apparatus

Ostwald-Folin pipettes accurately calibrated "to contain" 2 ml.

Round-bottom boiling flasks, 50 ml; fitted with a 1-hole rubber stopper and bent glass delivery tube.

Glass-stoppered cylinders, 25 ml in 0.5 ml; the 20 ml graduation must be accurately calibrated.

Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.

Reagents

1. **Sulfuric Acid, Volumetric N/12.** Add 2.5 ml concentrated sulfuric acid (H_2SO_4—Grasselli, A.C.S.) to 1000 ml distilled water and mix well; titrate against standard 0.1 N alkali using phenolphthalein as indicator; adjust accordingly and re-titrate—20 ml of N/12 acid require 16.7 ml of 0.1 N alkali.
2. **Sodium Tungstate, 10% Aqueous.** Dissolve 10 gm sodium tungstate ($Na_2WO_4 \cdot 2H_2O$—Mallinckrodt, Folin Special) in distilled water and dilute to 100 ml. The solution must be neutral or slightly alkaline to phenolphthalein, not more than 0.4 ml being required to neutralize 10 ml 0.1 N hydrochloric acid. Preserve in resistant-glass bottle.
3. **Sulfuric Acid, 50% by Volume.** Place 250 ml distilled water in a 600 ml beaker and immerse in ice water; add, slowly and with constant stirring, 250 ml concentrated sulfuric acid (H_2SO_4—Grasselli, A.C.S.); allow to cool to room temperature; transfer to a 500 ml volumetric flask and adjust to volume with distilled water. Preserve in Pyrex glass-stoppered bottle.
4. **Potassium Dichromate Solution.** Dry finely pulverized potassium dichromate ($K_2Cr_2O_7$—Reagent Chemicals, Special) at 110° C. for 2 hours; transfer 3.1943 gm to a 1000 ml volumetric flask; add approximately 500 ml distilled water and shake until dissolved; dilute to volume. Preserve in brown glass bottle.

Technic

1. In a 30 ml Erlenmeyer flask place
   16 ml N/12 sulfuric acid (1).
2. Add 2 ml oxalated whole blood from an Ostwald-Folin pipette, thoroughly rinsing out the pipette by drawing in and expelling some of the mixture several times.
3. Mix the contents of the flask by gentle rotation until the fluid is uniformly black-brown.
4. Add 2 ml 10% sodium tungstate (2), drop by drop while constantly, but gently, rotating the flask.
5. Shake the flask vigorously until the mixture is chocolate colored and foam-free.
6. Filter through a dry 9 cm Whatman #4 paper into a dry test tube.
7. In a round-bottom 50 ml boiling flask place 10 ml protein-free filtrate.
8. In a 25 ml glass-stoppered cylinder (sample) place
   5 ml 50% sulfuric acid (3)
   2 ml potassium dichromate solution (4), added from a volumetric pipette, and mix by lateral shaking.
9. Insert a 1-hole rubber stopper with delivery tube in the boiling flask—the short end extending about one-half inch below the stopper and the other end reaching nearly to the bottom of the 25 ml cylinder which has been placed in a 400 ml beaker containing cracked ice (See Fig. E-10).
10. Gently heat the flask with a free flame and distil the filtrate until the volume of fluid in the cylinder is about 14 ml.
11. Withdraw the glass tube from the cylinder and wash off the end with 1–2 ml distilled water from a wash bottle; make certain that all fluid inside the tube is expelled and then remove the flame.
12. Insert glass stopper in cylinder, sealing with a drop of concentrated sulfuric acid.
13. In another 25 ml glass-stoppered cylinder (reagent blank) place
    5 ml 50% sulfuric acid (3)
    2 ml potassium dichromate solution (4), added from a volumetric pipette
    10 ml distilled water and mix.
14. Place stoppered cylinders in water bath at 80° C. for 20 minutes.

15. Without removing the stoppers, cool to room temperature in running tap water.
16. Dilute to 20 ml with distilled water and mix.

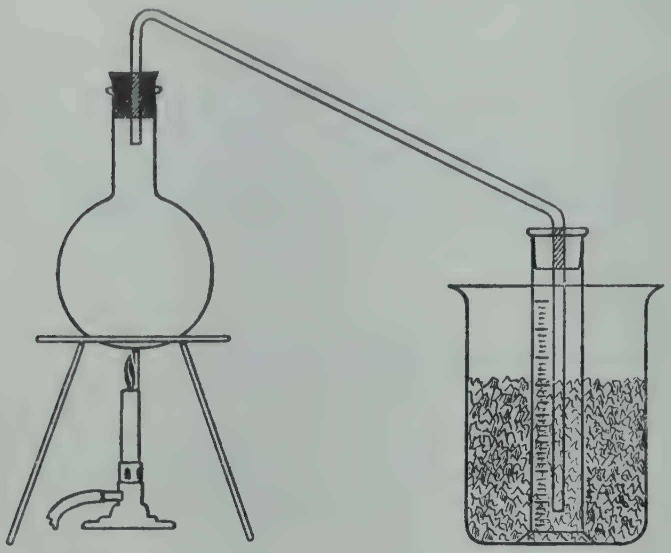

Fig. E-10.

17. Measure the transmittancy of the sample against the reagent blank set at *40%* transmission at a wave length of 450 mμ and obtain the concentration of ethyl alcohol from the calibration table.

CALIBRATION PROCEDURE

Special Apparatus

Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.

Glass-stoppered cylinders, 25 ml in 0.5 ml; the 20 ml graduation must be accurately calibrated.

Reagents

3. **Sulfuric Acid, 50% by Volume.** Same as for Test Procedure.
4. **Potassium Dichromate Solution.** Same as for Test Procedure.
5. **Ethyl Alcohol Standards**
   (a) Ethyl alcohol stock standard (1 ml contains 3 mg ethyl alcohol). Weigh 3 gm absolute ethyl alcohol (C_2H_5OH—U. S. Industrial Chemicals, U.S.P.) in a small glass-stoppered weighing bottle; transfer quantitatively to a 1000 ml volumetric flask and dilute to volume with distilled water. Note: The absolute ethyl alcohol should be kept in a glass-stoppered bottle over a layer of anhydrous copper sulfate.
   (b) Dilute standard (1 ml contains 0.15 mg ethyl alcohol). In a 100 ml volumetric flask place 5 ml stock standard (5a) and dilute to volume with distilled water. Prepare just before use.
   (c) Calibration standards. In each of a series of seven accurately calibrated 25 ml glass-stoppered cylinders place 5 ml 50% sulfuric acid (3), 2 ml potassium dichromate solution (4) and mix thoroughly; add respectively 0, 1, 3, 5, 7, 9 and 10 ml of the dilute standard (5b); dilute contents of each cylinder to approximately 17 ml with distilled water and mix. These standards represent concentrations of ethyl alcohol equivalent to 0, 15, 45, 75, 105, 135 and 150 mg per 100 ml sample. Prepare just before use and proceed immediately as follows.

Technic

1. Seal the glass stoppers with concentrated sulfuric acid.
2. Proceed exactly as outlined in the Test Procedure, Steps 14–16 inclusive.
3. Set the zero concentration standard at *40%* transmission at a wave length of 450 mμ and record the transmittancy of the 15, 45, 75, 105, 135 and 150 mg per cent standards. The transmittancy of the 150 mg standard should be approximately 73%.
4. Plot the observed values on semi-log graph paper—the points fall on a smooth curve which slopes upward and intersects the transmission axis at 40% (Calibration Curve E-10).
5. Prepare a calibration table from the T-C values obtained from the plotted curve.

Method E-10b

DETERMINATION IN URINE

SAMPLE

A casual specimen is usually sufficient for the purpose; for metabolic studies, use an aliquot of a 24-hour specimen kept cold during collection.

TEST PROCEDURE

Special Apparatus

As listed under the Test Procedure for blood.

Reagents

3. **Sulfuric Acid, 50% by Volume.** Same as for blood.
4. **Potassium Dichromate Solution.** Same as for blood.

Technic

1. Filter a small portion of thoroughly mixed urine.

2. In a 100 ml volumetric flask place 10 ml filtered urine and dilute to volume with distilled water.
3. In a round-bottom 50 ml boiling flask place 10 ml diluted urine.
4. Proceed exactly as outlined in the Test Procedure for blood, Steps 8–16 inclusive.
5. Measure the transmittancy of the sample against the reagent blank set at 40% transmission at a wave length of **450** mμ and obtain the concentration of ethyl alcohol directly from the calibration table for blood.

PROCEDURE FOR USE WITH LINEAR-SCALE PHOTOELECTRIC COLORIMETERS (SHEARD-SANFORD TYPE)

TEST PROCEDURE

Exactly as outlined; set the reagent blank at 40 and use a blue filter.

CALIBRATION PROCEDURE

Exactly as outlined—use a blue filter; plot the observed readings on semi-log graph paper—the points fall on a smooth curve which intersects the transmission axis at 40% and slopes upward.

PROCEDURE FOR USE WITH LOGARITHMIC-SCALE PHOTOELECTRIC COLORIMETERS (KLETT-SUMMERSON TYPE)

TEST PROCEDURE

Exactly as outlined; set the reagent blank at 230 and use a blue filter.

CALIBRATION PROCEDURE

Exactly as outlined—use a blue filter; plot the observed readings on linear graph paper—the points fall on a smooth curve which intersects the instrument reading axis at 230 and slopes downward.

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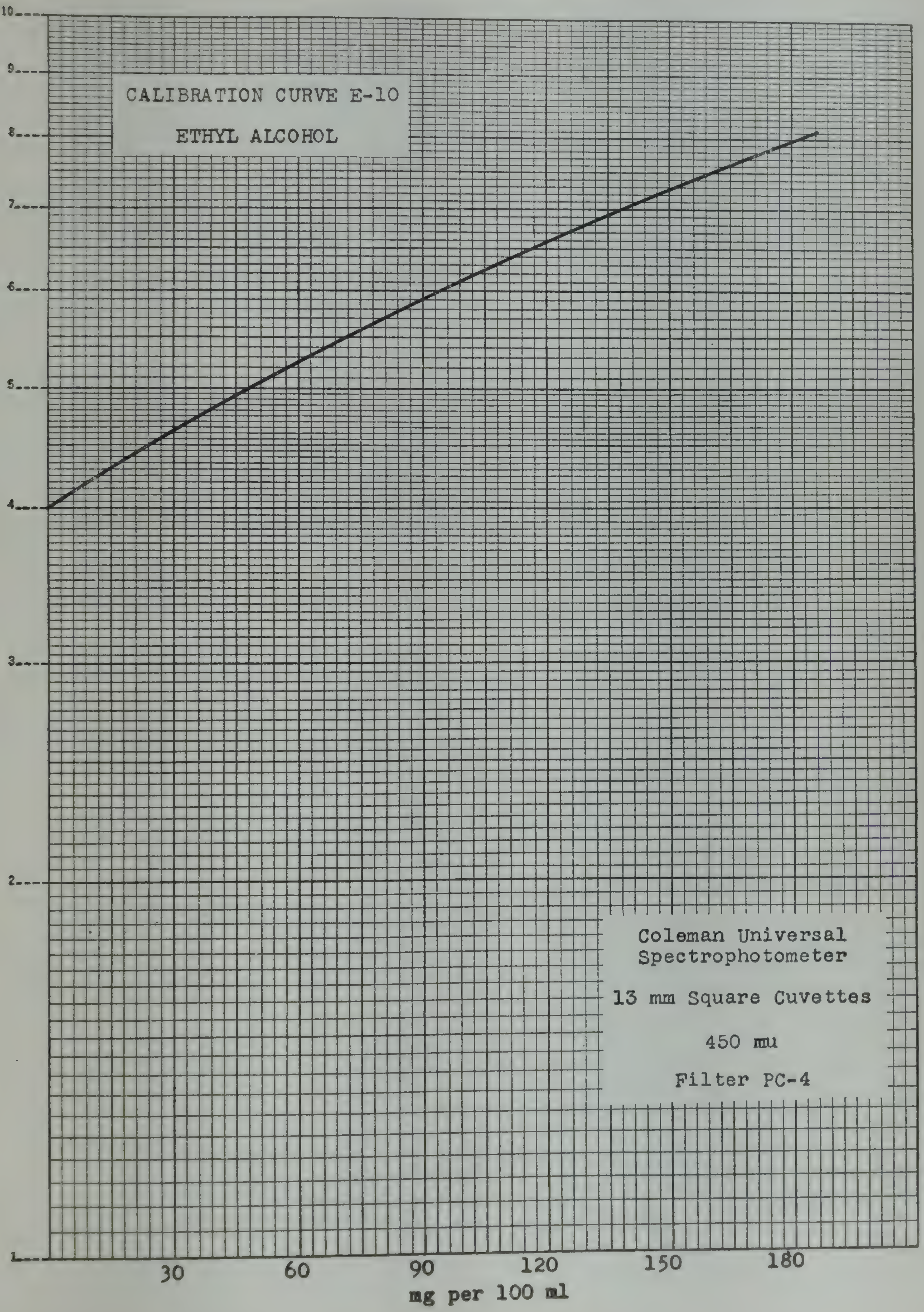
CALIBRATION CURVE E-10
ETHYL ALCOHOL
Coleman Universal Spectrophotometer
13 mm Square Cuvettes
450 mu
Filter PC-4
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8
7
6
5
4
3
2
1
30
60
90
120
150
180
mg per 100 ml

Meter Reading	0	1	2	3	4	5	6	7	8	9
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FIBRINOGEN

A spectrophotometric adaptation based on the methods and modifications of Cullen and Van Slyke (1920), Wu (1922), Wu and Ling (1927), Garbutt and Hubbard (1935), Kingsley (1939; 1940), Robinson and Hogden (1940), Hill and Trevorrow (1941), Weichselbaum (1946) and Gornall, Bardawill and David (1949)

PRINCIPLE

Fibrinogen is precipitated by calcium chloride, dissolved in alkali and determined by treatment with copper sulfate in the presence of alkaline sodium potassium tartrate to form a reddish-purple complex (biuret reaction).

DETERMINATION IN PLASMA

SAMPLE

Plasma from blood withdrawn during post-absorptive state. Anticoagulant—potassium oxalate; at least 5 mg of oxalate should be used for each milliliter of whole blood. There must be no trace of hemolysis. The plasma must be separated from the red cells as soon as possible.

TEST PROCEDURE

Special Apparatus

Glass-stoppered centrifuge tubes—Pyrex, 50 ml.

Ostwald-Folin pipettes accurately calibrated "to contain" 1 ml.

Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.

Reagents

1. **Sodium Chloride, 0.85% Aqueous.** Dissolve 8.5 gm sodium chloride ($NaCl$—Merck, Special for Biological Work) in distilled water and dilute to 1000 ml.
2. **Calcium Chloride, 2.5% Aqueous.** Dissolve 2.5 gm anhydrous calcium chloride ($CaCl_2$—J. T. Baker, A.C.S., For reagent use) or 5 gm crystalline calcium chloride ($CaCl_2 \cdot 6H_2O$—Merck, Reagent Neutral Crystals) in distilled water and dilute to 100 ml.
3. **Calcium Chloride, 0.05% Aqueous.** Dilute 2 ml 2.5% calcium chloride (2) to 100 ml with distilled water.
4. **Sodium Hydroxide, Volumetric 2.5 N.** Prepare by diluting slightly more than the required amount of concentrated carbonate-free sodium hydroxide solution (see Appendix); dilute 20 ml of this approximately 2.5 N solution to 100 ml; titrate against standard 0.5 N acid using phenolphthalein as indicator; adjust accordingly and re-titrate—20 ml of 0.5 N acid require 20 ml of a 1:5 dilution of 2.5 N alkali. Preserve in ceresin-lined bottle.
5. **Sulfuric Acid, Volumetric 2.5 N.** Slowly add 75 ml concentrated sulfuric acid (H_2SO_4—Grasselli, A.C.S.) to 750 ml distilled water; mix well and allow to cool; dilute 20 ml of this approximately 2.5 N solution to 100 ml; titrate against standard 0.5 N alkali using phenolphthalein as indicator; adjust accordingly and re-titrate—20 ml of a 1:5 dilution of 2.5 N acid require 20 ml of 0.5 N alkali.
6. **Biuret Reagent (Gornall-Bardawill-David).** In a 1000 ml volumetric flask place 1.5 gm crystalline copper sulfate ($CuSO_4 \cdot 5H_2O$—Baker & Adamson, A.C.S., Fine Crystals) and 6 gm sodium potassium tartrate ($NaKC_4H_4O_6 \cdot 4H_2O$—Baker & Adamson, A.C.S.); add approximately 500 ml distilled water and shake until dissolved; add, with constant agitation of the flask, 300 ml 2.5 N sodium hydroxide (4) and mix; add 1 gm potassium iodide (KI—Mallinckrodt, A.C.S.) and shake until dissolved; dilute to volume with distilled water. Preserve in ceresin-lined bottle. Stability of a properly prepared reagent is indefinite—discard if a black or reddish precipitate forms.

Technic

Note: For concentrations of fibrinogen in excess of 1000 mg per 100 ml, add 3.2 ml 0.85% sodium chloride to the neutralized fibrinogen solution in Step 12 and transfer exactly 2 ml of this dilute solution to an 18 × 150 mm Pyrex test tube before proceeding with Step 13 and multiply the values from the calibration table for total protein (Method P-50) by 200 to obtain the concentration of fibrinogen in mg per 100 ml; or multiply the values from the calibration table for fibrinogen by 2.

1. In a 50 ml glass-stoppered Pyrex centrifuge tube place
    - 23 ml 0.85% sodium chloride (1)
    - 1 ml plasma from an Ostwald-Folin pipette, thoroughly rinsing out the pipette by drawing in and expelling some of the mixture several times
    - 1 ml 2.5% calcium chloride (2).
2. Stopper the tube and mix by inversion.
3. Allow to stand for 3 hours.
4. Centrifuge at 2000 rpm for 15 minutes until the fibrin clot is tightly packed.
5. Decant the supernatant fluid and discard.
6. Loosen the clot with a thin glass rod and add 5 ml 0.05% calcium chloride (3), washing off the rod at the same time.
7. Centrifuge at 1800 rpm for 10 minutes, decant the supernatant fluid and discard.
8. Repeat Steps 6–7 inclusive, invert the tube on a

piece of filter paper and allow to drain for 10 minutes.

9. Add exactly 0.4 ml 2.5 N sodium hydroxide (4) and heat in water bath until fibrin clot is dissolved—calcium oxalate remains in suspension.
10. Add exactly 0.4 ml 2.5 N sulfuric acid (5) and mix.
11. Cool under running tap water for 3 minutes.
12. Add exactly 1.2 ml 0.85% sodium chloride (1) and mix.
13. Add 8 ml biuret reagent (6), mix by whirling and transfer contents to an 18 × 150 mm Pyrex test tube (sample) through a small filter paper.
14. In another 18 × 150 mm Pyrex test tube (reagent blank) place
    0.4 ml 2.5 N sodium hydroxide (4)
    0.4 ml 2.5 N sulfuric acid (5)
    1.2 ml 0.85% sodium chloride (1)
    8 ml biuret reagent (6) and mix by whirling; transfer to another tube through a small filter paper.
15. Place tubes in water bath at 25° C. for 30 minutes.
16. Measure the transmittancy of the sample against the reagent blank set at 100% transmission at a wave length of 540 mμ and multiply the value from the calibration table for total protein (Method P-50) by 100 to obtain the concentration of fibrinogen in mg per 100 ml. If a calibration table has been prepared for fibrinogen, the concentration is obtained directly.

CALIBRATION PROCEDURE

Note: The following calibration procedure is optional. The values in the calibration table for total protein (Method P-50), when multiplied by 100 are equivalent to the concentration of fibrinogen in mg per 100 ml.

Special Apparatus

Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.

Reagents

1. **Sodium Chloride, 0.85% Aqueous.** Same as for Test Procedure.
6. **Biuret Reagent (Gornall-Bardawill-David).** Same as for Test Procedure.
7. **Fibrinogen Standards**
    (a) Protein stock standard. Determine the total protein in a well mixed sample of pooled human sera by the Kjeldahl procedure (Method P-51).
    (b) Calibration standards. In a series of four accurately calibrated 100 ml volumetric flasks place respectively volumes of pooled sera (7a) containing 120, 240, 360 and 480 mg protein; dilute to volume with 0.85% sodium chloride (1). These standards represent concentrations of fibrinogen equivalent to 240, 480, 720 and 960 mg per 100 ml. Prepare just before use.

Technic

1. Transfer 2 ml of each calibration standard (7b) to a correspondingly marked 18 × 150 mm Pyrex test tube.
2. In a fifth 18 × 150 mm Pyrex test tube (zero concentration standard) place 2 ml 0.85% sodium chloride (1).
3. To each tube add 8 ml biuret reagent (6) and mix by whirling.
4. Place tubes in water bath at 25° C. for 30 minutes.
5. Remove tubes from water bath and immediately set the zero concentration standard at 100% transmission at a wave length of 540 mμ and record the transmittancy of the 240, 480, 720 and 960 mg per cent standards. The transmittancy of the 960 mg standard should be approximately 45.5%.
6. Plot the observed values on semi-log graph paper—the points fall on a straight line which passes through the origin (Calibration Curve F-10).
7. Prepare a calibration table from the T-C values obtained from the plotted curve.

PROCEDURE FOR USE WITH LINEAR-SCALE PHOTOELECTRIC COLORIMETERS (SHEARD-SANFORD TYPE)

TEST PROCEDURE

Exactly as outlined; set the reagent blank at 100 and use a green filter.

CALIBRATION PROCEDURE

Exactly as outlined—use a green filter; plot the observed readings on semi-log graph paper—the points fall on a straight line passing through the origin.

PROCEDURE FOR USE WITH LOGARITHMIC-SCALE PHOTOELECTRIC COLORIMETERS (KLETT-SUMMERSON TYPE)

TEST PROCEDURE

Exactly as outlined; set the reagent blank at 0 and use a green filter.

CALIBRATION PROCEDURE

Exactly as outlined—use a green filter; plot the observed readings on linear graph paper—the points fall on a straight line passing through the origin.

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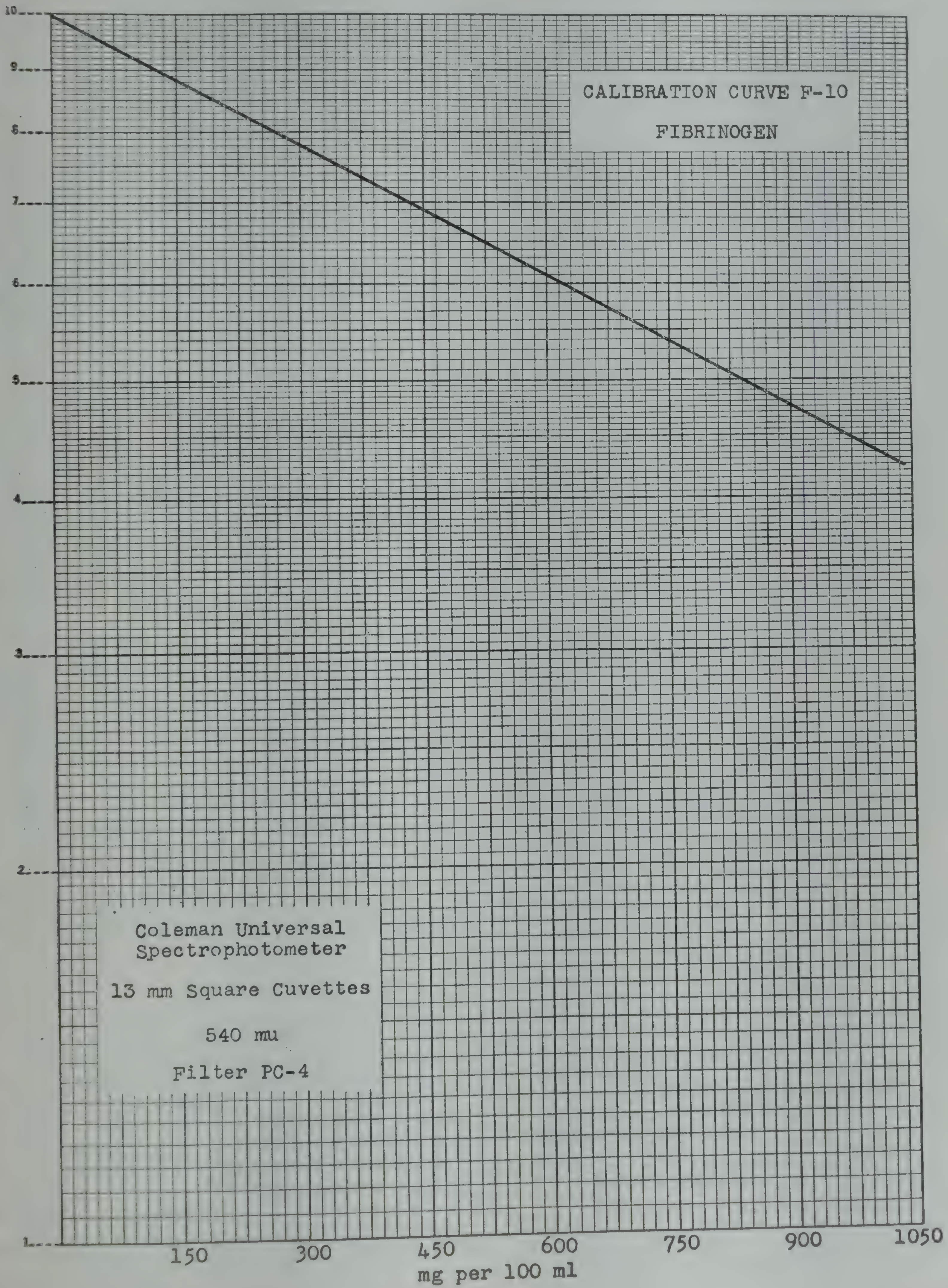
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FIBRINOGEN
Coleman Universal
Spectrophotometer
13 mm Square Cuvettes
540 mu
Filter PC-4
10
9
8
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6
5
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1
150
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600
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mg per 100 ml

Meter Reading	0	1	2	3	4	5	6	7	8	9
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FIBRINOGEN

A spectrophotometric adaptation based on the methods and modifications of Folin and Wu (1919), Cullen and Van Slyke (1920), Myers (1921), Garbutt and Hubbard (1935), Campbell and Hanna (1937), Hoffman and Osgood (1940) and Hill and Trevorrow (1941)

PRINCIPLE

Fibrinogen is precipitated by calcium chloride, dissolved in alkali and digested with a selenium-sulfuric acid mixture with the aid of hydrogen peroxide to convert nitrogenous constituents to ammonium sulfate in which the ammonia is determined by means of the reddish-orange color developed with alkaline potassium mercuric iodide (Nessler reaction).

DETERMINATION IN PLASMA

SAMPLE

Plasma from blood withdrawn during post-absorptive state. Anticoagulant—potassium oxalate; at least 5 mg of oxalate should be used for each milliliter of whole blood. There must be no trace of hemolysis. The plasma must be separated from the red blood cells as soon as possible. Contamination by ammonia or its salts must be avoided.

TEST PROCEDURE

Special Apparatus

Glass-stoppered centrifuge tubes—Pyrex, 50 ml.

Ostwald-Folin pipettes accurately calibrated "to contain" 1 ml.

Fister digestion tubes—Pyrex; 25 × 200 mm; glass-stoppered, the 20 ml graduation must be accurately calibrated. These tubes prevent contamination during mixing as well as protect the hands from the action of the alkali. Folin-Wu tubes may be used provided that they are accurately calibrated at 20 ml.

Micro burner, Argand type.

Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.

Reagents

1. **Water, Distilled, Ammonia-Free.** In an all-glass Pyrex distillation apparatus place 1 gm sodium carbonate (Na_2CO_3—Baker & Adamson, A.C.S.) and 1 gm potassium permanganate ($KMnO_4$—Baker & Adamson, A.C.S.); add 500 ml distilled water; redistil, rejecting the first 100 ml portion and collecting only the next 300 ml. Use for the preparation of all reagents and standards and where indicated in the test procedure.
2. **Sodium Chloride, 0.85% Aqueous.** Dissolve 8.5 gm sodium chloride (NaCl—Merck, Special for Biological Work) in ammonia-free distilled water (1) and dilute to 1000 ml.
3. **Calcium Chloride, 2.5% Aqueous.** Dissolve 2.5 gm anhydrous calcium chloride ($CaCl_2$—J. T. Baker, A.C.S., For reagent use) or 5 gm crystalline calcium chloride ($CaCl_2 \cdot 6H_2O$—Merck, Reagent Neutral Crystals) in ammonia-free distilled water (1) and dilute to 100 ml.
4. **Calcium Chloride, 0.05% Aqueous.** Dilute 2 ml 2.5% calcium chloride (3) to 100 ml with ammonia-free distilled water (1).
5. **Sodium Hydroxide, Volumetric 2.5 N.** Prepare by diluting slightly more than the required amount of concentrated carbonate-free sodium hydroxide solution (see Appendix); dilute 20 ml of this approximately 2.5 N solution to 100 ml; titrate against standard 0.5 N acid using phenolphthalein as indicator; adjust accordingly and re-titrate—20 ml of 0.5 N acid require 20 ml of a 1:5 dilution of 2.5 N alkali. Preserve in ceresin-lined bottle.
6. **Selenium-Sulfuric Acid Digestion Mixture "C" (Fister).** In a 1000 ml Florence flask place 450 ml ammonia-free distilled water (1); add slowly, while cooling the flask under running tap water, 17.6 ml concentrated sulfuric acid (H_2SO_4—Grasselli, A.C.S.); cool to room temperature; transfer to a 500 ml mixing cylinder; add 0.15 ml selenium oxychloride ($SeOCl_2$—J. T. Baker); dilute to volume with ammonia-free distilled water and mix thoroughly. Preserve in Pyrex glass-stoppered bottle. Note: This is not the same digestion mixture used in the nonprotein nitrogen and total nitrogen determinations.
7. **Hydrogen Peroxide, 30%** (H_2O_2—Merck, Reagent Superoxol, Low-Nitrogen).
8. **Nessler Reagent (Folin-Wu).** In a 1000 ml mixing cylinder place 700 ml 2.5 N sodium hydroxide (5); add 150 ml double iodide solution (a); dilute to volume with ammonia-free distilled water (1) and mix; allow to stand for three days to permit sedimentation of the small amount of precipitate which forms. Preserve in ceresin-lined bottle. Stability indefinite. Note: The alkaline concentration of this reagent is 7%—11.0–11.5 ml must be required to neutralize 20 ml 1 N hydrochloric acid, using phenolphthalein as indicator.
   (a) Double iodide solution (Folin-Wu); in a 500 ml Florence flask place 150 gm granular potassium iodide (KI—Mallinckrodt, A.C.S.) and 100 gm resublimed iodine (I—Baker & Adamson, A.C.S.); add 100 ml ammonia-free distilled water (1) and shake

until the iodine has completely dissolved; add 150 gm redistilled metallic mercury (Hg—Baker & Adamson, A.C.S.); shake the flask continuously and vigorously until the red color of the iodine solution has become visibly pale, though still red; immediately start cooling under running tap water, continuing vigorous shaking, until the reddish color has been replaced by the greenish-yellow color of the double iodide. If properly prepared, the solution is clear and there is a residue of shining metallic mercury; solutions showing a gray-green sludge are unsatisfactory. The whole operation should consume no more than 10–15 minutes. Since the reaction develops considerable heat, precautions should be taken to protect the hands during the shaking procedure. Decant the solution into a 2000 ml mixing cylinder, washing mercury and flask with liberal quantities of ammonia-free distilled water and decanting the washings into the cylinder. Dilute to volume with ammonia-free distilled water.

Technic

Note: For concentrations of fibrinogen nitrogen in excess of 100 mg per 100 ml, dilute one volume of diluted fibrinogen solution with an equal volume of ammonia-free distilled water (1) before proceeding with Step 14 and multiply the values obtained from the calibration table by 2.

1. In a 50 ml glass-stoppered Pyrex centrifuge tube place
   23 ml 0.85% sodium chloride (2)
   1 ml plasma from an Ostwald-Folin pipette, thoroughly rinsing out the pipette by drawing in and expelling some of the mixture several times
   1 ml 2.5% calcium chloride (3).
2. Stopper the tube and mix by inversion.
3. Allow to stand for 3 hours.
4. Centrifuge at 2000 rpm for 15 minutes until the fibrin clot is tightly packed.
5. Decant the supernatant fluid and discard.
6. Loosen the clot with a thin glass rod and add 5 ml 0.05% calcium chloride (4), washing off the rod at the same time.
7. Centrifuge at 1800 rpm for 10 minutes, decant the supernatant fluid and discard.
8. Repeat Steps 6–7 inclusive, invert the tube on a piece of filter paper and allow to drain for 10 minutes.
9. Add 0.4 ml 2.5 N sodium hydroxide (5) and heat in water bath until fibrin clot is dissolved—calcium oxalate remains in suspension.
10. Add 10 ml ammonia-free distilled water (1) and centrifuge at 1800 rpm for 10 minutes.
11. Without disturbing the sedimented calcium oxalate, transfer the centrifugate to a 50 ml volumetric flask.
12. Repeat Steps 10–11 inclusive and transfer the centrifugate to the flask.
13. Add 0.8 ml selenium-sulfuric acid digestion mixture "C" (6) to the centrifugate in the flask and dilute to volume with ammonia-free distilled water (1); mix.
14. In a special dry digestion tube (sample) place
    2.5 ml diluted fibrinogen solution from Step 13
    2 ml selenium-sulfuric acid digestion mixture "C" (6)
    1 solid glass bead.
15. Clamp tube in a vertical position on a support and apply gentle heat from an Argand micro burner in such a manner that the flame at no time touches the tube above the level of the liquid.

Note: Carry out Steps 15–21 inclusive in a fume hood.

16. After boiling has started gradually increase the heat and place burner in upright position so that the top is about $^3/_4$ inch below the bottom of the tube and the point of the flame directly under the tube—the bubbles should at no time rise above the middle of the tube.
17. Heat until white fumes of SO_3 fill about one-half of the tube—none of these fumes should escape.
18. Insert a small funnel into the mouth of the tube and continue gentle heating with reduced flame, until the charred digest begins to clear.
19. Remove the flame and allow to cool for 90 seconds.
20. Remove the funnel and allow 1 drop 30% hydrogen peroxide (7) to fall directly on the digest.
21. Replace funnel and heat gently for 2 minutes making certain that any charred material which may have adhered to the walls of the tube has been washed down and decomposed—the digest should be perfectly clear—if not, add another drop of hydrogen peroxide in the same manner and heat again until clear.
22. Without removing the funnel, allow to cool for 2 minutes and then place the tube in a beaker half-full of ice-cold water.
23. In another special digestion tube (reagent blank) place 2 ml selenium-sulfuric acid digestion mixture "C" (6) and place in the cold water bath with the other tube.
24. After 10 minutes in the cold water bath, add about 8 ml ammonia-free distilled water (1) to each tube, rinsing off the funnel in the sample tube at the same time and mix, allowing the tubes to remain in the bath.
25. Without removing the tubes from the bath add 10 ml Nessler reagent (8), allowing the reagent to fall directly into the solution.
26. Remove the tubes from the bath, dilute to 20 ml with ammonia-free distilled water (1), insert the glass stoppers and mix by inversion.
27. Replace the tubes in the bath and allow to remain for 5 minutes.

28. Remove the tubes from the bath and allow to stand at room temperature (21° C.) for 10 minutes.
29. Measure the transmittancy of the sample against the reagent blank set at 100% transmission at a wave length of **415** mμ and obtain the concentration of fibrinogen nitrogen from the calibration table.

Calculation

To determine the fibrinogen concentration—

Fibrinogen nitrogen in mg per 100 ml × 6.25 =
Concentration of fibrinogen in mg per 100 ml

CALIBRATION PROCEDURE

Special Apparatus

Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.

Fister digestion tubes, glass-stoppered as described in the Test Procedure.

Micro burner, Argand type.

Reagents

1. **Water, Distilled, Ammonia-Free.** Same as for Test Procedure.
6. **Selenium-Sulfuric Acid Digestion Mixture "C" (Fister).** Same as for Test Procedure.
7. **Hydrogen Peroxide, 30%.** Same as for Test Procedure.
8. **Nessler Reagent (Folin-Wu).** Same as for Test Procedure.
9. **Fibrinogen Standards**
   (a) Nitrogen stock standard (1 ml contains 10 mg nitrogen). Dry pyridine-free ammonium sulfate ($(NH_4)_2SO_4$—Reagent Chemicals, Special) at 110° C. for 1 hour; transfer 4.7166 gm to a 100 ml volumetric flask; add approximately 50 ml ammonia-free distilled water (1); shake until dissolved and dilute to volume with same. Preserve with a few drops of toluene. Stability indefinite.
   (b) Dilute standard (1 ml contains 0.1 mg nitrogen). In a 100 ml volumetric flask place 1 ml stock standard (9a) and dilute to volume with ammonia-free distilled water (1). Prepare just before use.
   (c) Calibration standards. In a series of six accurately calibrated 10 ml volumetric flasks place respectively 0, 1, 2, 3, 4 and 5 ml of the dilute standard (9b); dilute to volume with ammonia-free distilled water (1). These standards represent concentrations of nitrogen equivalent to 0, 20, 40, 60, 80 and 100 mg per 100 ml sample. Prepare just before use.

Technic

1. Into correspondingly marked special dry digestion tubes place
   1 ml of each calibration standard (9c)
   2 ml selenium-sulfuric acid digestion mixture "C" (6)
   1 solid glass bead.
2. Treat each tube exactly as outlined in the Test Procedure, Steps 15–28 inclusive—the standards are run consecutively through Steps 15–22 inclusive, Step 23 is omitted (since this duplicates the zero concentration calibration standard) and then simultaneously through Steps 24–28 inclusive.
3. Set the zero concentration standard at 100% transmission at a wave length of **415** mμ and record the transmittancy of the 20, 40, 60, 80 and 100 mg per cent standards. The transmittancy of the 100 mg standard should be approximately 24%.
4. Plot the observed values on semi-log graph paper—the points fall on a slight curve which passes through the concentration axis slightly to the right of the origin (Calibration Curve F-11).
5. Prepare a calibration table from the T-C values obtained from the plotted curve.

PROCEDURE FOR USE WITH LINEAR-SCALE PHOTOELECTRIC COLORIMETERS (SHEARD-SANFORD TYPE)

TEST PROCEDURE

Exactly as outlined; set the reagent blank at 100 and use a blue filter.

CALIBRATION PROCEDURE

Exactly as outlined—use a blue filter; plot the observed readings on semi-log graph paper—the points fall on a slight curve which passes through the concentration axis slightly to the right of the origin.

PROCEDURE FOR USE WITH LOGARITHMIC-SCALE PHOTOELECTRIC COLORIMETERS (KLETT-SUMMERSON TYPE)

TEST PROCEDURE

Exactly as outlined; set the reagent blank at 0 and use a blue filter.

CALIBRATION PROCEDURE

Exactly as outlined—use a blue filter; plot the observed readings on linear graph paper—the points fall on a slight curve which passes through the concentration axis slightly to the right of the origin.

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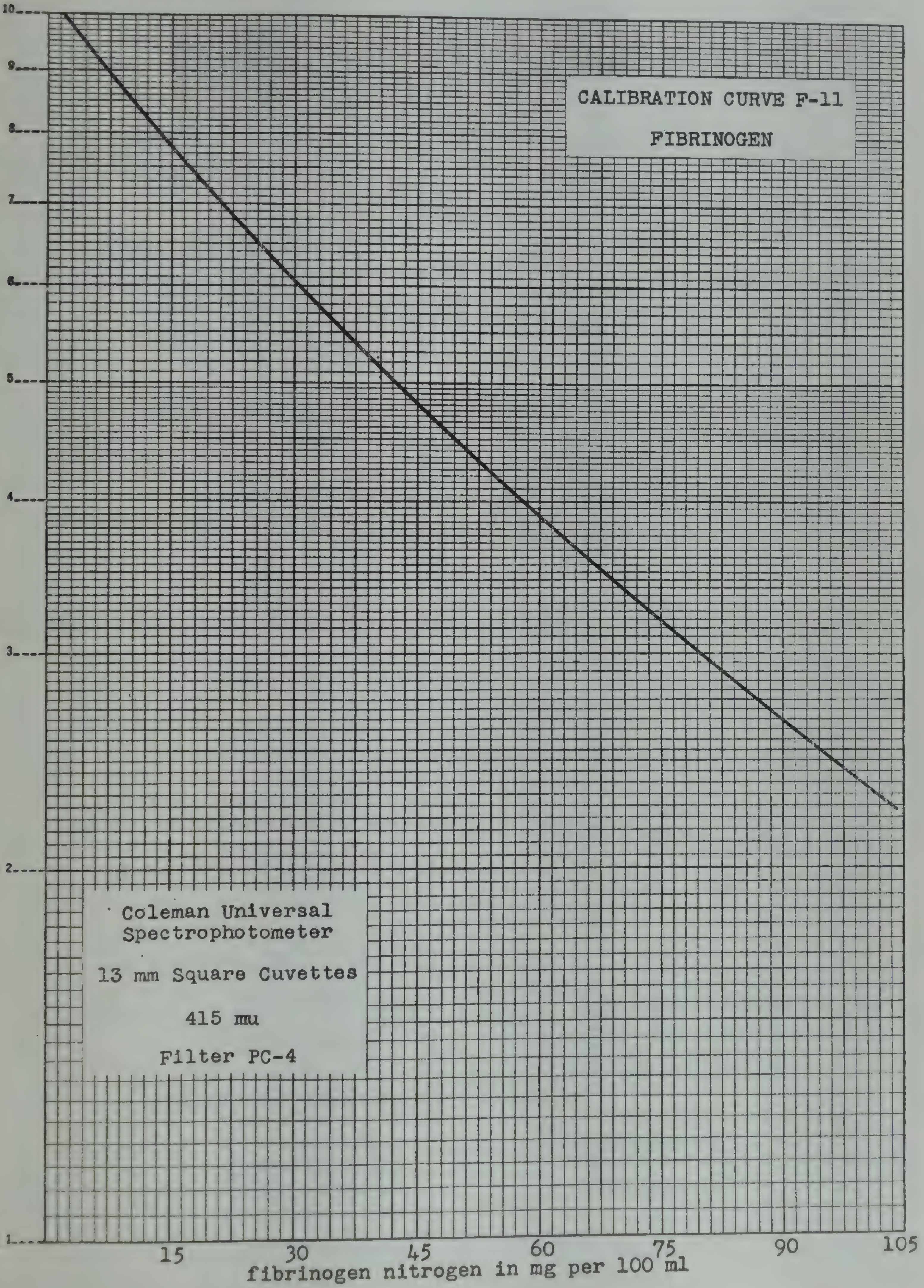
NOTES


CALIBRATION CURVE F-11
FIBRINOGEN
Coleman Universal Spectrophotometer
13 mm Square Cuvettes
415 mu
Filter PC-4
10
9
8
7
6
5
4
3
2
1
15
30
45
60
75
90
105
fibrinogen nitrogen in mg per 100 ml

Meter Reading	0	1	2	3	4	5	6	7	8	9
0										
10										
20										
30										
40										
50										
60										
70										
80										
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FIBRINOGEN

A spectrophotometric adaptation based on the methods and modifications of Cullen and Van Slyke (1920), Wu (1922), Wu and Ling (1927), Folin and Ciocalteu (1927), Greenberg (1929), Andersch and Gibson (1933), Garbutt and Hubbard (1935), Greenberg and Mirolubova (1936), Kingsley (1940), Maclay and Osterberg (1940) and Hill and Trevorrow (1941)

PRINCIPLE

Fibrinogen is precipitated by calcium chloride, dissolved in alkali and determined by means of the Folin-Ciocalteu phenol reagent as outlined in the method for total protein (Method P-52), the same analytical principles being involved. One milligram tyrosine produces a color equivalent to the color produced by 11.55 mg fibrinogen.

DETERMINATION IN PLASMA

SAMPLE

Plasma from blood withdrawn during post-absorptive state. Anticoagulant—potassium oxalate; at least 5 mg of oxalate should be used for each milliliter of whole blood. There must be no trace of hemolysis. The plasma must be separated from the red blood cells as soon as possible.

TEST PROCEDURE

Special Apparatus

Glass-stoppered centrifuge tubes—Pyrex, 50 ml.

Ostwald-Folin pipettes accurately calibrated "to contain" 0.5 ml.

Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.

Reagents

1. **Sodium Chloride, 0.85% Aqueous.** Dissolve 8.5 gm sodium chloride (NaCl—Merck, Special for Biological Work) in distilled water and dilute to 1000 ml.
2. **Calcium Chloride, 2.5% Aqueous.** Dissolve 2.5 gm anhydrous calcium chloride ($CaCl_2$—J. T. Baker, A.C.S., For reagent use) or 5 gm crystalline calcium chloride ($CaCl_2 \cdot 6H_2O$—Merck, Reagent Neutral Crystals) in distilled water and dilute to 100 ml.
3. **Calcium Chloride, 0.05% Aqueous.** Dilute 2 ml 2.5% calcium chloride (2) to 100 ml with distilled water.
4. **Sodium Hydroxide, Volumetric 2.5 N.** Prepare by diluting slightly more than the required amount of concentrated carbonate-free sodium hydroxide solution (see Appendix); dilute 20 ml of this approximately 2.5 N solution to 100 ml; titrate against standard 0.5 N acid using phenolphthalein as indicator; adjust accordingly and retitrate—20 ml of 0.5 N acid require 20 ml of a 1:5 dilution of 2.5 N alkali. Preserve in ceresin-lined bottle.
5. **Phenol Reagent (Folin-Ciocalteu).** In a 1500 ml boiling flask with ground joint place 500 ml distilled water; add 100 gm sodium tungstate ($Na_2WO_4 \cdot 2H_2O$—Mallinckrodt, Folin Special) and shake until dissolved; add 25 gm sodium molybdate ($Na_2MoO_4 \cdot 2H_2O$—Mallinckrodt, AR); add 200 ml distilled water, washing down the neck of the flask and shake until solution is complete; add 50 ml 85% phosphoric acid (H_3PO_4—Baker & Adamson, A.C.S.) and mix thoroughly; add 100 ml concentrated hydrochloric acid (HCl—Baker & Adamson, A.C.S.) and mix thoroughly; fit a Liebig reflux condenser to the flask and boil gently for 10 hours (during this period the solution changes in color from a pale yellow to a deep green); remove the reflux condenser; add 150 gm lithium sulfate ($LiSO_4$—Baker & Adamson, Reagent) and 50 ml distilled water, washing down the neck of the flask; shake thoroughly until completely dissolved; add 10 drops bromine (Br—Baker & Adamson, A.C.S.) and boil for 15 minutes to remove the excess bromine; allow to cool to room temperature; dilute to 1000 ml with distilled water and filter through tightly packed glass wool into a brown glass-stoppered bottle. The reagent should show no tint of green and if kept well protected from dust will remain stable for months. A reagent which develops a green color in the course of time may be repurified by the addition of a few drops of bromine and boiling as before.

Technic

Note: For concentrations of fibrinogen in excess of 750 mg per 100 ml, use 100 ml volumetric flasks in Steps 12 and 13 and multiply the values from the calibration table for total protein (Method P-52) by 100 to obtain the concentration of fibrinogen in mg per 100 ml; or multiply the values from the calibration table for fibrinogen by 2.

1. In a 50 ml glass-stoppered Pyrex centrifuge tube place
   24 ml 0.85% sodium chloride (1)
   0.5 ml 2.5% calcium chloride (2) and mix
   0.5 ml plasma from an Ostwald-Folin pipette, thoroughly rinsing out the pipette by drawing in and expelling some of the mixture several times.
2. Stopper the tube and mix the contents by inversion.

3. Allow to stand at room temperature for 3 hours.
4. Centrifuge at 2000 rpm for 15 minutes until the fibrin clot is tightly packed, decant the supernatant fluid and discard.
5. Loosen the clot with a thin glass rod and add 5 ml 0.05% calcium chloride (3), washing off the rod at the same time.
6. Centrifuge at 1800 rpm for 10 minutes, decant the supernatant fluid and discard.
7. Repeat Steps 5–6 inclusive, invert the tube on a piece of filter paper and allow to drain for 10 minutes.
8. Add 6 ml distilled water
   4 ml 2.5 N sodium hydroxide (4) and mix.
9. Place in boiling water for 10 minutes.
10. Cool in running tap water for 3 minutes.
11. Centrifuge at 1500 rpm for 10 minutes.
12. Decant the clear centrifugate into a 50 ml volumetric flask (sample); wash out the centrifuge tube with distilled water and transfer the washings to the flask through a small filter paper.
13. In another 50 ml volumetric flask (reagent blank) place 4 ml 2.5 N sodium hydroxide (4).
14. To each flask add about 25 ml distilled water and mix.
15. Add 3 ml phenol reagent (5), slowly and with constant agitation of the flask.
16. Dilute to volume with distilled water and mix thoroughly by inversion.
17. Allow to stand at room temperature for 10 minutes.
18. Measure the transmittancy of the sample against the reagent blank set at 100% transmission at a wave length of **700 mμ** and multiply the value from the calibration table for total protein (Method P-52) by 50 to obtain the concentration of fibrinogen in mg per 100 ml. If a calibration table has been prepared for fibrinogen, the concentration is obtained directly.

CALIBRATION PROCEDURE

Note: The following calibration procedure is optional. The values in the calibration table for total protein (Method P-52), when multiplied by 50, are equivalent to the concentration of fibrinogen in mg per 100 ml.

Special Apparatus

Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.

Reagents

4. **Sodium Hydroxide, Volumetric 2.5 N.** Same as for Test Procedure.
5. **Phenol Reagent (Folin-Ciocalteu).** Same as for Test Procedure.
6. **Hydrochloric Acid, Volumetric 0.1 N.** Add 10 ml concentrated hydrochloric acid (HCl—Baker & Adamson, A.C.S.) to 1000 ml distilled water and mix; standardize against 0.1 N alkali using phenolphthalein as indicator.
7. **Fibrinogen Standards**
   (a) Tyrosine stock standard (1 ml contains 0.2 mg tyrosine). In a 100 ml volumetric flask place 20 mg l-tyrosine ($C_9H_{11}O_3N$—Pfanstiehl, C.P.), add about 85 ml 0.1 N hydrochloric acid (6); shake until dissolved and dilute to volume with same. Preserve with a few drops of xylene.
   (b) Dilute standard (1 ml contains 0.05 mg tyrosine). In a 100 ml volumetric flask place 25 ml stock standard (7a) and dilute to volume with distilled water. Prepare just before use.
   (c) Calibration standards. In a series of six accurately calibrated 100 ml volumetric flasks place respectively 0, 2.5, 5, 7.5, 10 and 12.5 ml of the dilute standard (7b) and proceed as follows. These standards represent concentrations of fibrinogen equivalent to 0, 144, 288, 433, 578 and 722 mg per 100 ml sample. Prepare just before use.

Technic

1. To each calibration standard (7c) add 4 ml 2.5 N sodium hydroxide (4).
2. Proceed exactly as outlined in the Test Procedure, Steps 14–17 inclusive.
3. Set the zero concentration standard at 100% transmission at a wave length of **700 mμ** and record the transmittancy of the 144, 288, 433, 578 and 722 mg per cent standards. The transmittancy of the 722 mg standard should be approximately 27%.
4. Plot the observed values on semi-log graph paper—the points fall on a straight line which passes through the origin (Calibration Curve F-12).
5. Prepare a calibration table from the T-C values obtained from the plotted curve.

◆

PROCEDURE FOR USE WITH LINEAR-SCALE PHOTOELECTRIC COLORIMETERS (SHEARD-SANFORD TYPE)

TEST PROCEDURE

Exactly as outlined; set the reagent blank at 100 and use a red filter.

CALIBRATION PROCEDURE

Exactly as outlined—use a red filter; plot the observed readings on semi-log graph paper—the points fall on a straight line passing through the origin.

◆

PROCEDURE FOR USE WITH LOGARITHMIC-SCALE PHOTOELECTRIC COLORIMETERS (KLETT-SUMMERSON TYPE)

TEST PROCEDURE

Exactly as outlined; set the reagent blank at 0 and use a red filter.

CALIBRATION PROCEDURE

Exactly as outlined—use a red filter; plot the observed readings on linear graph paper—the points fall on a straight line passing through the origin.

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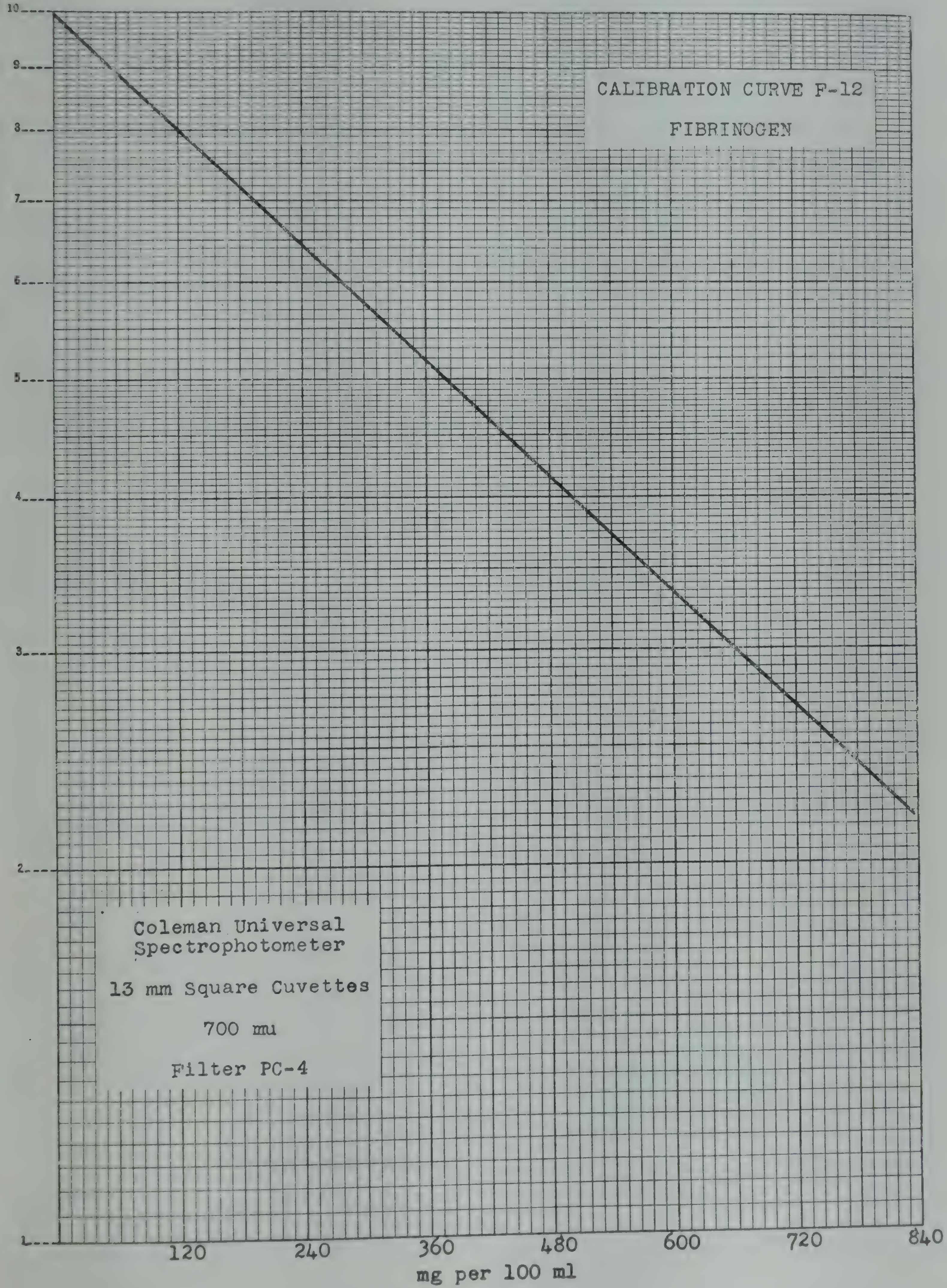
CALIBRATION CURVE F-12
FIBRINOGEN
Coleman Universal Spectrophotometer
13 mm Square Cuvettes
700 mu
Filter PC-4
10
9
8
7
6
5
4
3
2
1
120
240
360
480
600
720
840
mg per 100 ml

Meter Reading	0	1	2	3	4	5	6	7	8	9
0										
10										
20										
30										
40										
50										
60										
70										
80										
90										

GALACTOSE

A spectrophotometric adaptation based on the methods and modifications of Bauer (1906), Folin and Wu (1919), Somogyi (1927; 1928), Raymond and Blanco (1928), Jankelson, Segal and Aisner (1937), Shay and Fieman (1937), Althausen, Lockhart and Soley (1940), Nelson (1944) and Somogyi (1945)

PRINCIPLE

Dextrose is removed from the blood by fermentation with yeast. The galactose is then determined in the protein-free filtrate by copper reduction as outlined in the method for true dextrose (Method D-12a), the same analytical principles being involved. Galactose has a lower reducing power than dextrose, the color developed by dextrose being approximately one and one-quarter times that developed by an equivalent amount of galactose.

DETERMINATION IN WHOLE BLOOD, PLASMA OR SERUM

SAMPLE

When carrying out the galactose tolerance test, three or more samples are necessary—one from blood withdrawn during post-absorptive state prior to the administration of galactose and two or more, depending upon the technic followed, from blood secured at definite intervals after the administration of the sugar. Anticoagulant—potassium oxalate; a fluoride-oxalate-thymol mixture (Reagent #1) is preferable if determinations cannot be made without undue delay. Prepare protein-free filtrate at once and keep in refrigerator until use.

Special Note: The accuracy of the galactose tolerance test depends upon the purity of the galactose used for both administration in the test procedure and for the preparation of calibration solutions. Only anhydrous d(+)galactose with a specific rotation of at least +80° should be used—commercial galactose with a specific rotation of +70° to +76° is not satisfactory.

TEST PROCEDURE

Special Apparatus

Ostwald-Folin pipettes accurately calibrated "to contain" 2 ml.

Folin sugar tubes, accurately graduated at the 15 ml level. For detailed specifications see the remarks in Method D-10a.

Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.

Reagents

1. **Fluoride-Oxalate-Thymol Mixture (Levinson-MacFate).** In a mortar place 10 gm powdered neutral sodium fluoride (NaF—Baker & Adamson, A.C.S.), 1 gm powdered thymol ($C_{10}H_{14}O$—Merck, Reagent) and 3 gm powdered neutral potassium oxalate ($K_2C_2O_4 \cdot H_2O$—J. T. Baker, A.C.S.); grind thoroughly until the components are intimately mixed. Fifteen milligrams are sufficient for the prevention of coagulation and the preservation of five milliliters of blood.
2. **Yeast Suspension.** In a glass mortar place 10 gm fresh commercial yeast (Standard Brands) and triturate with a small portion of distilled water to form a thick paste; transfer to a 100 ml mixing cylinder, washing out the mortar with further small portions of water and transferring the washings to the cylinder; dilute to volume and shake thoroughly until completely suspended; transfer the suspension to two 50 ml centrifuge tubes and centrifuge at 2000 rpm for 5 minutes; discard the supernatant fluid; add about 40 ml distilled water to each tube and stir up the sediment thoroughly; again centrifuge and discard the supernatant fluid and repeat the washing until the supernatant fluid is clear and colorless; drain tubes in an inverted position for 3 minutes; add 25 ml distilled water to each tube, shake thoroughly and combine the suspensions. Preserve in refrigerator. Stability approximately 2 weeks—however, a blank analysis should be run occasionally and if the presence of reducing substances is shown, a fresh suspension should be prepared.
3. **Tungstic Acid Solution.** Just before use, mix equal volumes 2/3 N sulfuric acid (a) and 10% sodium tungstate (b).
    (a) Sulfuric acid, volumetric 2/3 N; slowly add 20 ml concentrated sulfuric acid (H_2SO_4—Grasselli, A.C.S.) to 1000 ml distilled water and mix well; titrate against standard 1 N alkali using phenolphthalein as indicator; adjust accordingly and re-titrate —20 ml of 2/3 N acid require 13.3 ml of 1 N alkali.
    (b) Sodium tungstate, 10% aqueous; dissolve 10 gm sodium tungstate ($Na_2WO_4 \cdot 2H_2O$—Mallinckrodt, Folin Special) in distilled water and dilute to 100 ml. The solution must be neutral or slightly alkaline to phenolphthalein, not more than 0.4 ml being required to neutralize 10 ml 0.1 N hydrochloric acid. Preserve in resistant-glass bottle.
4. **Copper-Phosphate-Tartrate Reagent, High Alkalinity (Somogyi).** In a 1500 ml beaker place 700 ml distilled water; add 28 gm anhydrous dibasic sodium phosphate (Na_2HPO_4—Mallinckrodt, AR) and stir until dissolved; add 40 gm crystalline sodium potassium tartrate

($NaKC_4H_4O_6 \cdot 4H_2O$—Baker & Adamson, A.C.S.) and stir until dissolved; add 100 ml volumetric 1 N sodium hydroxide (a) and mix; add 80 ml 10% copper sulfate (b) with constant stirring; add 180 gm anhydrous sodium sulfate (Na_2SO_4—Baker & Adamson, Reagent Special); stir until solution is complete; transfer to a 1000 ml mixing cylinder and dilute to volume with distilled water; allow to stand for 2 days until any sediment has settled; filter thru Whatman #4 paper. Preserve in resistant-glass bottle. This reagent is stable indefinitely and shows no deposition of cuprous oxide.

(a) Sodium hydroxide, volumetric 1 N; prepare by diluting slightly more than the required amount of concentrated carbonate-free sodium hydroxide solution (see Appendix) and standardize against 1 N acid using phenolphthalein as indicator. Preserve in ceresin-lined bottle.

(b) Copper sulfate, 10% aqueous; dissolve 10 gm finely crystalline copper sulfate ($CuSO_4 \cdot 5H_2O$—Baker & Adamson, A.C.S., Fine Crystals) in distilled water and dilute to 100 ml.

5. **Arsenomolybdate Color Reagent (Nelson).** In a 1000 ml Florence flask place 25 gm ammonium molybdate ($(NH_4)_6Mo_7O_{24} \cdot 4H_2O$—Mallinckrodt, AR) and dissolve in 450 ml distilled water; add 21 ml concentrated sulfuric acid (H_2SO_4—Grasselli, A.C.S.) and mix; dissolve 3 gm crystalline sodium acid arsenate ($Na_2HAsO_4 \cdot 7H_2O$—Baker & Adamson, Reagent) in 25 ml distilled water and add to the contents of the flask; mix and place in incubator at 37° C. for 48 hours. Preserve in brown glass-stoppered bottle.

Technic

Note: For concentrations of galactose in excess of 150 mg per 100 ml, use 1 ml protein-free centrifugate (or filtrate) and 1 ml distilled water in Step 8 and multiply the values obtained from the calibration table by 2.

1. In a 50 ml Erlenmeyer flask (sample blank) place
   14 ml yeast suspension (2)
   2 ml sample from blood withdrawn prior to the administration of galactose, from an Ostwald-Folin pipette, thoroughly rinsing out the pipette by drawing in and expelling some of the mixture several times, and mix.
2. In another 50 ml Erlenmeyer flask (sample 1) place
   14 ml yeast suspension (2)
   2 ml sample from the first blood specimen withdrawn after the administration of galactose, from an Ostwald-Folin pipette in the same manner as before, and mix.
3. In similar 50 ml Erlenmeyer flasks place 14 ml yeast suspension (2) and 2 ml sample from each additional blood specimen.
4. Allow the flasks to stand at room temperature for 20 minutes.
5. To each flask add 4 ml tungstic acid solution (3) and shake vigorously.
6. Allow to stand for 5 minutes.
7. Transfer the contents of each flask to correspondingly marked 25 ml centrifuge tubes and centrifuge at 1800 rpm for 5 minutes (or filter through dry 11 cm Whatman #40 papers into dry test tubes)—the centrifugates (or filtrates) must be absolutely clear.
8. Place 2 ml of each clear supernatant centrifugate (or filtrate) in a correspondingly marked Folin sugar tube, placing the tip of the pipette near the bottom of the tube so that the fluid does not flow down the walls.
9. To each tube add 2 ml copper-phosphate-tartrate reagent (4) and mix by lateral shaking.
10. Place the tubes in boiling water for 20 minutes.
11. Cool under running tap water for 3 minutes.
12. Add 2 ml arsenomolybdate color reagent (5), allowing the reagent to flow rapidly down the side of the tube and mix by vigorous lateral shaking.
13. Allow to stand at room temperature for 2 minutes.
14. Dilute to 15 ml with distilled water and mix by inverting the tubes three times, allowing the bulbs to empty each time.
15. Measure the transmittancy of the samples against the sample blank set at 100% transmission at a wave length of **500 mμ** and obtain the concentrations of galactose from the calibration table.

Note: The concentration of galactose may be obtained by multiplying the values from the calibration table for true dextrose (Method D-12A) by 0.7.

CALIBRATION PROCEDURE

Special Apparatus

Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.

Folin sugar tubes, accurately graduated at the 15 ml level.

Reagents

4. **Copper-Phosphate-Tartrate Reagent, High Alkalinity (Somogyi).** Same as for Test Procedure.
5. **Arsenomolybdate Color Reagent (Nelson).** Same as for Test Procedure.
6. **Benzoic Acid, 0.25% Aqueous.** In a 1500 ml beaker heat 1000 ml distilled water to boiling; add 2.5 gm benzoic acid ($C_7H_6O_2$—Baker & Adamson, Reagent) and stir until dissolved; allow to cool; adjust volume to 1000 ml with distilled water and filter.
7. **Galactose Standards**

   (a) Galactose stock standard (1 ml contains 10 mg galactose). Dry galactose ($C_6H_{12}O_6$—Reagent Chemicals, Special according to Bauer) over sulfuric acid in vacuo to constant weight; transfer 1 gm to a 100 ml volumetric flask; add 50 ml 0.25% benzoic acid (6); shake until dissolved; dilute to volume with same and mix. Stability indefinite.

(b) Dilute standard (1 ml contains 1 mg galactose). In a 100 ml volumetric flask place 10 ml stock standard (7a); dilute to volume with 0.25% benzoic acid (6) and mix. Stable for approximately one month in refrigerator.

(c) Calibration standards. In a series of seven accurately calibrated 100 ml volumetric flasks place respectively 0, 2.5, 5.0, 7.5, 10, 12.5 and 15 ml of the dilute standard (7b) and dilute to volume with distilled water; mix. These standards represent concentrations of galactose equivalent to 0, 25, 50, 75, 100, 125 and 150 mg per 100 ml sample. Prepare just before use.

Technic

1. Transfer 2 ml of each calibration standard (7c) to a correspondingly marked Folin sugar tube.
2. Proceed exactly as outlined in the Test Procedure, Steps 9–14 inclusive.
3. Set the zero concentration standard at 100% transmission at a wave length of 500 mμ and record the transmittancy of the 25, 50, 75, 100, 125 and 150 mg per cent standards. The transmittancy of the 150 mg standard should be approximately 23%.
4. Plot the observed values on semi-log graph paper—the points fall on a straight line which passes through the origin (Calibration Curve G-10).
5. Prepare a calibration table from the T-C values obtained from the plotted curve.

Method G-10b

DETERMINATION IN URINE

SAMPLE

First morning fasting specimen and additional specimens as required, usually one every hour for 4 hours after the administration of galactose. Determinations should be made without undue delay or the specimen should be preserved under a layer of toluene and kept cold until use.

TEST PROCEDURE

Special Apparatus

Ostwald-Folin pipettes accurately calibrated "to contain" 2 ml.

Folin sugar tubes, accurately graduated at the 15 ml level.

Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.

Reagents

2. **Yeast Suspension.** Same as for blood.
3. **Tungstic Acid Solution.** Same as for blood.
4. **Copper-Phosphate-Tartrate Reagent, High Alkalinity (Somogyi).** Same as for blood.
5. **Arsenomolybdate Color Reagent (Nelson).** Same as for blood.
8. **Oxalic Acid, Volumetric 0.05 N.** In a 1000 ml volumetric flask place 3.1518 gm crystalline oxalic acid ($H_2C_2O_4 \cdot 2H_2O$—J. T. Baker, Special for Standardizing); add 800 ml distilled water and shake until dissolved; dilute to volume with distilled water.
9. **Lloyd's Reagent (Lilly).** Other suitable adsorbents may be used.

Technic

Note: For concentrations of galactose in excess of 3%, dilute 1 ml filtrate to 20 ml with distilled water in Step 7 and multiply the values obtained from the calibration table for blood by 40.

1. In a 50 ml Erlenmeyer flask (sample blank) place
   10 ml 0.05 N oxalic acid (8)
   10 ml thoroughly mixed fasting urine specimen.
2. In another 50 ml Erlenmeyer flask (sample I) place
   10 ml 0.05 N oxalic acid (8)
   10 ml thoroughly mixed urine voided 1 hour after the administration of galactose.
3. In similar 50 ml Erlenmeyer flasks place 10 ml 0.05 N oxalic acid (8) and 10 ml thoroughly mixed urine from each additional specimen.
4. To each flask add 1.5 gm Lloyd's reagent (9).
5. Stopper the flasks and shake vigorously for 3 minutes.
6. Filter through dry papers into correspondingly marked dry test tubes.
7. Dilute 1 ml of each filtrate to 10 ml with distilled water.
8. In each of a series of 50 ml Erlenmeyer flasks place
   14 ml yeast suspension (2).
9. In the first flask (sample blank) place
   2 ml of the corresponding filtrate from an Ostwald-Folin pipette, thoroughly rinsing out the pipette by drawing in and expelling some of the mixture several times, and mix.
10. Transfer 2 ml of each additional filtrate to a correspondingly marked flask in the same manner as above.
11. Allow to stand at room temperature for 20 minutes.

12. Proceed exactly as outlined in the Test Procedure for blood, Steps 5–14 inclusive.
13. Measure the transmittancy of the samples against the sample blank set at 100% transmission at a wave length of **500** mμ and multiply the values from the calibration table for blood by 20 to obtain the concentration of galactose in mg per 100 ml urine.

Calculation

To convert galactose in mg per 100 ml to per cent—

$$\frac{\text{Galactose in mg per 100 ml}}{1000} = \text{Galactose in \%}$$

$$\frac{\text{Total volume of urine voided during test period}}{100} \times \text{Galactose in \%} = \text{Gm galactose excreted}$$

PROCEDURE FOR USE WITH LINEAR-SCALE PHOTOELECTRIC COLORIMETERS (SHEARD-SANFORD TYPE)

TEST PROCEDURE

Exactly as outlined; set the sample blank at 100 and use a blue filter.

CALIBRATION PROCEDURE

Exactly as outlined—use a blue filter; plot the observed readings on semi-log graph paper—the points fall on a straight line passing through the origin.

PROCEDURE FOR USE WITH LOGARITHMIC-SCALE PHOTOELECTRIC COLORIMETERS (KLETT-SUMMERSON TYPE)

TEST PROCEDURE

Exactly as outlined; set the sample blank at 0 and use a blue filter.

CALIBRATION PROCEDURE

Exactly as outlined—use a blue filter; plot the observed readings on linear graph paper—the points fall on a straight line passing through the origin.

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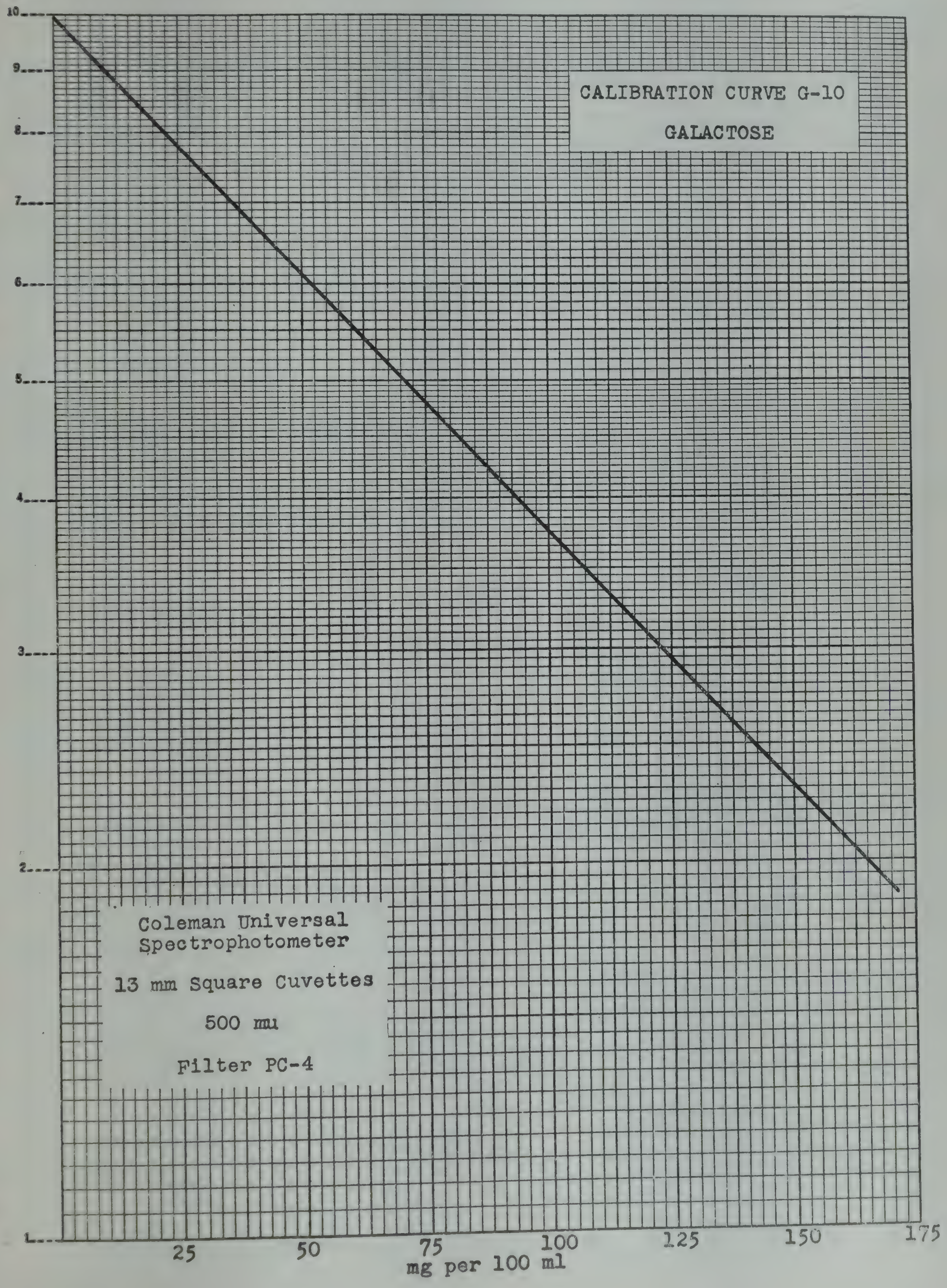
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13 mm Square Cuvettes
500 mμ
Filter PC-4
10
9
8
7
6
5
4
3
2
1
25
50
75
100
125
150
175
mg per 100 ml

Meter Reading	0	1	2	3	4	5	6	7	8	9
0										
10										
20										
30										
40										
50										
60										
70										
80										
90										

GLOBULIN

A spectrophotometric adaptation based on the methods and modifications of Kingsley (1939; 1940; 1942), Maclay and Osterberg (1940), Robinson and Hogden (1940), Weichselbaum (1946) and Gornall, Bardawill and David (1949)

Either of two methods may be used for the estimation of globulin—by difference or by actual determination

A. By Difference

1. Determine the concentration of albumin as outlined in Method A-10.
2. Determine the concentration of fibrinogen as outlined in Method F-10 and divide the value in mg per 100 ml by 1000 to convert to per cent.
3. Determine the concentration of total protein as outlined in Method P-50.
4. Calculation—

Total protein in % − (albumin in % + fibrinogen in %) = globulin in %

Note: The determination of fibrinogen in routine practice is usually omitted and not corrected for in the calculation.

B. By Determination

PRINCIPLE

Globulin is precipitated by sodium sulfate, the density of which is decreased by the use of ether so that the precipitated globulin forms in a compact mass at the interface between the two layers. The globulin is dissolved in sodium chloride and determined by treatment with copper sulfate in the presence of alkaline sodium potassium tartrate to form a reddish-purple complex (biuret reaction).

DETERMINATION IN PLASMA OR SERUM

SAMPLE

Plasma or serum from blood withdrawn during postabsorptive state. Anticoagulant—potassium oxalate. There must be no trace of hemolysis. Plasma or serum must be separated from the red blood cells as soon as possible.

TEST PROCEDURE

Special Apparatus

Glass-stoppered centrifuge tubes, 15 ml.
Ostwald-Folin pipettes accurately calibrated "to contain" 0.5 ml.
Hooked nichrome wire embedded in a glass rod.
Graduated test tubes—10 ml in 0.1 ml; the 5 ml graduation must be accurately calibrated.
Wash bottle, 125 ml with capillary tip.
Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.

Reagents

1. **Sodium Sulfate, 23% Aqueous.** Heat approximately 200 ml distilled water to 37° C.; half fill a 100 ml volumetric flask with the warm water; add 23 gm anhydrous sodium sulfate (Na_2SO_4—Baker & Adamson, Reagent Special); add more warm water until the body of the flask is just about filled; stopper and shake until solution is complete; dilute to volume with the warm water. Keep in incubator at 37° C.

Note: Reagent grade sodium sulfate may vary considerably in its free acid or free alkali content. Each new lot should be tested as follows: dissolve 5 gm in 60 ml carbon dioxide-free distilled water and add 3 drops phenolphthalein indicator solution—no pink color should appear; add 0.5 ml 0.02 N sodium hydroxide—a pink color should appear. Sodium sulfate which does not show these characteristics is unsatisfactory for the differential precipitation of proteins and should be discarded—the reaction of the final solution must be just on the acid side of neutrality.

2. **Ether, Anhydrous** ($(C_2H_5)_2O$—Mallinckrodt, A.C.S.).
3. **Sodium Chloride, 0.85% Aqueous.** Dissolve 8.5 gm sodium chloride (NaCl—Merck, Special for Biological Work) in distilled water and dilute to 1000 ml.
4. **Biuret Reagent (Gornall-Bardawill-David).** In a 1000 ml volumetric flask place 1.5 gm crystalline copper sulfate ($CuSO_4 \cdot 5H_2O$—Baker & Adamson, A.C.S., Fine Crystals) and 6 gm sodium potassium tartrate ($NaKC_4H_4O_6 \cdot 4H_2O$—Baker & Adamson, A.C.S.); add approximately 500 ml distilled water and shake until dissolved; add, with constant agitation of the flask, 300 ml 2.5 N sodium hydroxide (a) and mix; add 1 gm potassium iodide (KI—Mallinckrodt, A.C.S.) and shake until dissolved; dilute to volume with distilled water. Preserve in ceresin-lined bottle. Stability of a properly prepared reagent is indefinite—discard if a black or reddish precipitate forms.
   (a) Sodium hydroxide, volumetric 2.5 N; prepare by diluting slightly more than the required amount of concentrated carbonate-free sodium hydroxide solution (see Appendix); dilute 20 ml of this approximately 2.5 N solution to 100 ml; titrate against standard 0.5 N acid using phenolphthalein as indicator; adjust accordingly and re-titrate—20 ml of 0.5 N acid require 20 ml of a 1:5 dilution of 2.5 N alkali. Preserve in ceresin-lined bottle.

Technic

Note: If globulin is to be estimated by actual determination on the precipitate as reserved in the albumin analysis (Method A-10, Step 7) omit the following Steps 1–6 inclusive.

1. Place a 15 ml glass-stoppered centrifuge tube in a 150 ml beaker half-filled with warm water at 37° C.
2. Without removing the tube from the beaker, add 7.5 ml 23% sodium sulfate (1), removed from the incubator immediately before use.
3. Add 0.5 ml sample from a warm Ostwald-Folin pipette, thoroughly rinsing out the pipette by drawing in and expelling some of the mixture several times.
4. Stopper the tube and mix the contents by inversion.
5. Add 4 ml ether (2), stopper the tube, and shake vigorously for 30 seconds.
6. Centrifuge the unstoppered tube at 2000 rpm for 15 minutes—the precipitated globulin is tightly packed in a layer at the interface between the sulfate solution and the ether.
7. Slant the tube so that the globulin layer is separated from the walls; with a hooked nichrome wire remove the precipitated globulin en masse and transfer to a small dry Whatman #40 filter paper in a dry funnel. RESERVE SULFATE LAYER FOR ALBUMIN ANALYSIS (Method A-10). Note: If the compact layer should disintegrate, remove as much of the ether as possible by means of a capillary pipette and filter the entire contents of the tube through a small dry Whatman #40 paper, reserving a portion of the filtrate for the albumin analysis before proceeding with the next step.
8. Wash the globulin precipitate with three 3 ml portions 23% sodium sulfate (1)—in the case of a disintegrated precipitate rinse out the centrifuge tube with three 3 ml portions 23% sodium sulfate, pouring the washings over the precipitate on the paper—discard the washings.
9. Insert the funnel into a 10 ml graduated test tube and puncture a small hole in the tip of the paper.
10. Wash all the globulin precipitate from the paper into the tube by means of small portions of 0.85% sodium chloride (3) delivered forcibly in a very fine stream from a capillary-tipped wash bottle.
11. Open the filter paper and make certain that all the globulin has been washed into the tube—the total volume in the tube should not exceed 5 ml.
12. Place in boiling water for 5 minutes until all the globulin precipitate is dissolved.
13. Cool in running tap water for 3 minutes.
14. Adjust volume to 5 ml with distilled water and mix.
15. In an 18 × 150 mm Pyrex test tube (sample) place
    2 ml diluted globulin solution from Step 14.
16. In another 18 × 150 mm Pyrex test tube (reagent blank) place
    2 ml 0.85% sodium chloride (3).
17. To each tube add 8 ml biuret reagent (4) and mix by whirling.
18. Place tubes in water bath at 25° C. for 30 minutes.
19. Measure the transmittancy of the sample against the reagent blank set at 100% transmission at a wave length of 540 mμ and multiply the value from the calibration table for total protein (Method P-50) by 0.5 to obtain the concentration of globulin in per cent. If a calibration table has been prepared for globulin, the concentration is obtained directly.

CALIBRATION PROCEDURE

Note: The following calibration procedure is optional. The values in the calibration table for total protein (Method P-50), when multiplied by 0.5 are equivalent to the concentration of globulin in per cent.

Special Apparatus

Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.

Reagents

3. **Sodium Chloride, 0.85% Aqueous.** Same as for Test Procedure.
4. **Biuret Reagent (Gornall-Bardawill-David).** Same as for Test Procedure.
5. **Globulin Standards**
    (a) Protein stock standard. Determine the total protein in a well mixed sample of pooled human sera by the Kjeldahl procedure (Method P-51).
    (b) Calibration standards. In a series of four accurately calibrated 100 ml volumetric flasks place respectively volumes of pooled sera (5a) containing 120, 240, 360 and 480 mg protein; dilute to volume with 0.85% sodium chloride (3). These standards represent concentrations of globulin equivalent to 1.2, 2.4, 3.6 and 4.8%. Prepare just before use.

Technic

1. Transfer 2 ml of each calibration standard (5b) to a correspondingly marked 18 × 150 mm Pyrex test tube.
2. Proceed exactly as outlined in the Test Procedure, Steps 16–18 inclusive.
3. Remove tubes from water bath and immediately set the zero concentration standard at 100% transmission at a wave length of 540 mμ and record the transmittancy of the 1.2, 2.4, 3.6 and 4.8% standards. The transmittancy of the 4.8% standard should be approximately 45.5%.
4. Plot the observed values on semi-log graph paper—

the points fall on a straight line which passes through the origin (Calibration Curve G-15).

5. Prepare a calibration table from the T-C values obtained from the plotted curve.

PROCEDURE FOR USE WITH LINEAR-SCALE PHOTOELECTRIC COLORIMETERS (SHEARD-SANFORD TYPE)

TEST PROCEDURE

Exactly as outlined; set the reagent blank at 100 and use a green filter.

CALIBRATION PROCEDURE

Exactly as outlined—use a green filter; plot the observed readings on semi-log graph paper—the points fall on a straight line passing through the origin.

PROCEDURE FOR USE WITH LOGARITHMIC-SCALE PHOTOELECTRIC COLORIMETERS (KLETT-SUMMERSON TYPE)

TEST PROCEDURE

Exactly as outlined; set the reagent blank at 0 and use a green filter.

CALIBRATION PROCEDURE

Exactly as outlined—use a green filter; plot the observed readings on linear graph paper—the points fall on a straight line passing through the origin.

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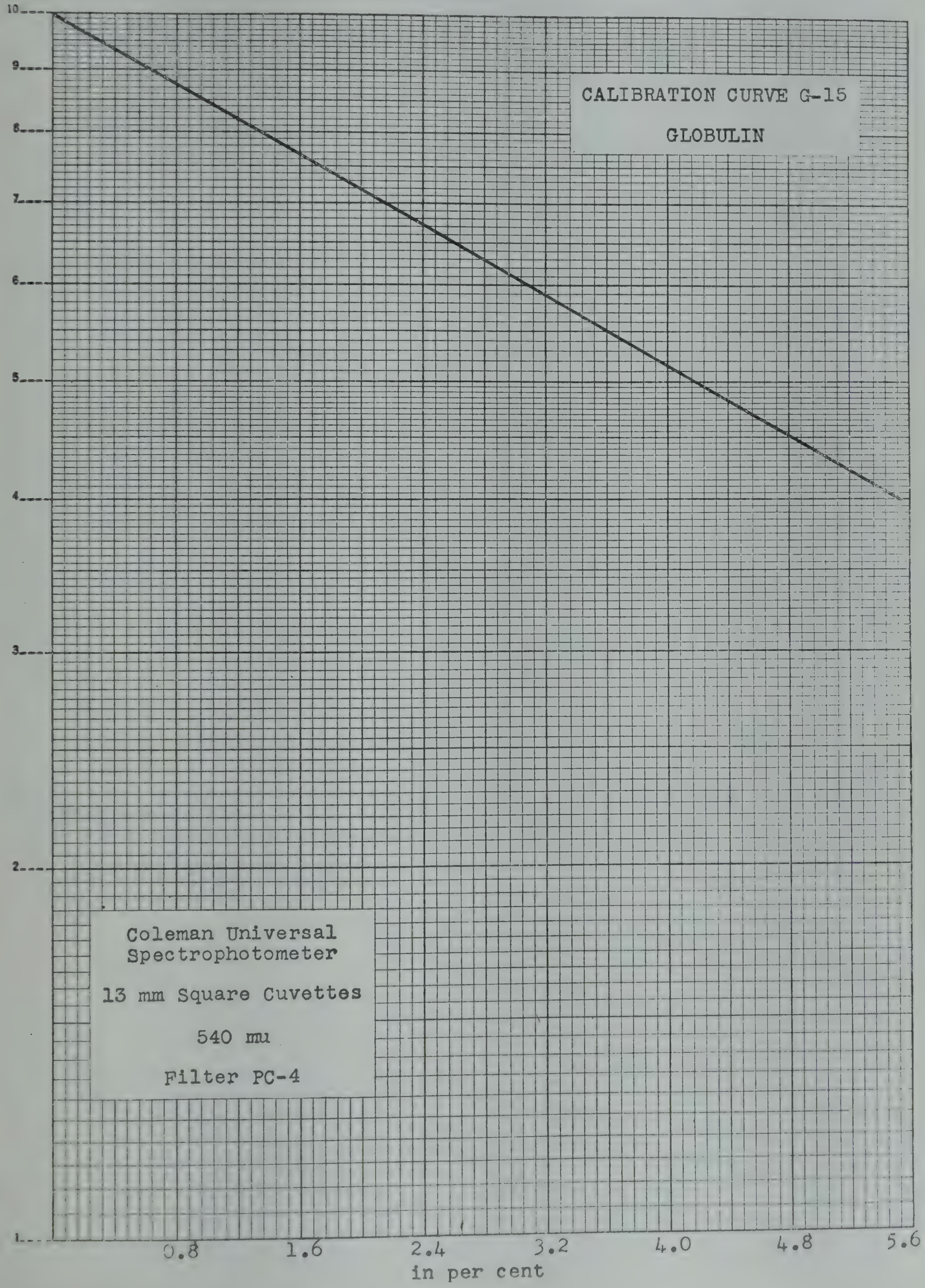
NOTES

NOTES


CALIBRATION CURVE G-15
GLOBULIN
Coleman Universal Spectrophotometer
13 mm Square Cuvettes
540 mu
Filter PC-4
10
9
8
7
6
5
4
3
2
1
0.8
1.6
2.4
3.2
4.0
4.8
5.6
in per cent

Meter Reading	0	1	2	3	4	5	6	7	8	9
0										
10										
20										
30										
40										
50										
60										
70										
80										
90										

GLOBULIN

A spectrophotometric adaptation based on the methods and modifications of Folin and Wu (1919), Howe (1921), Myers (1921), Campbell and Hanna (1937), Hoffman and Osgood (1940), Kingsley (1940) and Maclay and Osterberg (1940)

Either of two methods may be used for the estimation of globulin—by difference or by actual determination.

A. By Difference

1. Determine the concentration of albumin as outlined in Method A-11.
2. Determine the concentration of fibrinogen as outlined in Method F-11 and divide the value in mg per 100 ml by 1000 to convert to per cent.
3. Determine the concentration of total protein as outlined in Method P-51.
4. Calculation—

Total protein in % − (albumin in % + fibrinogen in %) = globulin in %

Note: The determination of fibrinogen in routine practice is usually omitted and not corrected for in the calculation.

B. By Determination

PRINCIPLE

Globulin is precipitated by sodium sulfate, the density of which is decreased by the use of ether so that the precipitated globulin forms in a compact mass at the interface of the two layers. The globulin is dissolved in sodium hydroxide and digested with a selenium-sulfuric acid mixture with the aid of hydrogen peroxide to convert nitrogenous constituents to ammonium sulfate in which the ammonia is determined by means of the reddish-orange color developed with alkaline potassium mercuric iodide (Nessler reaction).

DETERMINATION IN PLASMA OR SERUM

SAMPLE

Plasma or serum from blood withdrawn during post-absorptive state. Anticoagulant—potassium oxalate. There must be no trace of hemolysis. Plasma or serum must be separated from the red blood cells as soon as possible. Contamination by ammonia or its salts must be avoided.

TEST PROCEDURE

Special Apparatus

Glass-stoppered centrifuge tubes, 15 ml.
Ostwald-Folin pipettes accurately calibrated "to contain" 0.5 ml.
Hooked nichrome wire embedded in a glass rod.
Fister digestion tubes—Pyrex; 25 × 200 mm; glass-stoppered; the 20 ml graduation must be accurately calibrated. These tubes prevent contamination during mixing as well as protect the hands from the action of the alkali. Folin-Wu tubes may be used provided that they are accurately calibrated at 20 ml.
Micro burner, Argand type.
Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.

Reagents

1. **Water, Distilled, Ammonia-Free.** In an all-glass Pyrex distillation apparatus place 1 gm sodium carbonate (Na_2CO_3—Baker & Adamson, A.C.S.) and 1 gm potassium permanganate ($KMnO_4$—Baker & Adamson, A.C.S.); add 500 ml distilled water; redistil, rejecting the first 100 ml portion and collecting only the next 300 ml. Use for the preparation of all reagents and standards and where indicated in the test procedure.
2. **Sodium Sulfate, 23% Aqueous.** Heat approximately 200 ml ammonia-free distilled water (1) to 37° C.; half fill a 100 ml volumetric flask with the warm water; add 23 gm anhydrous sodium sulfate (Na_2SO_4—Baker & Adamson, Reagent Special); add more warm water until the body of the flask is just about filled; stopper and shake until solution is complete; dilute to volume with the warm water. Keep in incubator at 37° C.

   Note: Reagent grade sodium sulfate may vary considerably in its free acid or free alkali content. Each new lot should be tested as follows: dissolve 5 gm in 60 ml carbon dioxide-free distilled water and add 3 drops phenolphthalein indicator solution—no pink color should appear; add 0.5 ml 0.02 N sodium hydroxide—a pink color should appear. Sodium sulfate which does not show these changes is unsatisfactory for the differential precipitation of proteins and should be discarded—the reaction of the final solution must be just on the acid side of neutrality.
3. **Ether, Anhydrous** ($(C_2H_5)_2O$—Mallinckrodt, A.C.S.).
4. **Sodium Chloride, 0.85% Aqueous.** Dissolve 8.5 gm sodium chloride (NaCl—Merck, Special for Biological Work) in ammonia-free distilled water (1) and dilute to 1000 ml.
5. **Sodium Hydroxide, Volumetric 2.5 N.** Prepare by diluting slightly more than the required amount of concentrated carbonate-free sodium hydroxide solution (see Appendix); dilute 20 ml of this approximately 2.5 N solution to 100 ml; titrate against standard 0.5 N acid using phenolphthalein as indicator; adjust accordingly and re-titrate—20 ml of 0.5 N acid require 20 ml of a

1:5 dilution of 2.5 N alkali. Preserve in ceresin-lined bottle.

6. **Selenium-Sulfuric Acid Digestion Mixture "C" (Fister).** In a 1000 ml Florence flask place 450 ml ammonia-free distilled water (1); add slowly, while cooling the flask under running tap water, 17.6 ml concentrated sulfuric acid (H_2SO_4—Grasselli, A.C.S.); cool to room temperature; transfer to a 500 ml mixing cylinder; add 0.15 ml selenium oxychloride ($SeOCl_2$—J. T. Baker); dilute to volume with ammonia-free distilled water and mix thoroughly. Preserve in Pyrex glass-stoppered bottle. Note: This is not the same digestion mixture used in the nonprotein nitrogen and total nitrogen determinations.
7. **Hydrogen Peroxide, 30%** (H_2O_2—Merck, Reagent Superoxol, Low-Nitrogen).
8. **Nessler Reagent (Folin-Wu).** In a 1000 ml mixing cylinder place 700 ml 2.5 N sodium hydroxide (5); add 150 ml double iodide solution (a); dilute to volume with ammonia-free distilled water (1) and mix; allow to stand for three days to permit sedimentation of the small amount of precipitate which forms. Preserve in ceresin-lined bottle. Stability indefinite. Note: The alkaline concentration of this reagent is 7%—11.0–11.5 ml must be required to neutralize 20 ml 1 N hydrochloric acid, using phenolphthalein as indicator.
   (a) Double iodide solution (Folin-Wu); in a 500 ml Florence flask place 150 gm granular potassium iodide (KI—Mallinckrodt, A.C.S.) and 100 gm resublimed iodine (I—Baker & Adamson, A.C.S.); add 100 ml ammonia-free distilled water (1) and shake until the iodine has completely dissolved; add 150 gm redistilled metallic mercury (Hg—Baker & Adamson, A.C.S.); shake the flask continuously and vigorously until the red color of the iodine solution has become visibly pale, though still red; immediately start cooling under running tap water, continuing vigorous shaking, until the reddish color has been replaced by the greenish-yellow color of the double iodide. If properly prepared, the solution is clear and there is a residue of shining metallic mercury; solutions showing a gray-green sludge are unsatisfactory. The whole operation should consume no more than 10–15 minutes. Since the reaction develops considerable heat, precautions should be taken to protect the hands during the shaking procedure. Decant the solution into a 2000 ml mixing cylinder, washing mercury and flask with liberal quantities of ammonia-free distilled water and decanting the washings into the cylinder. Dilute to volume with ammonia-free distilled water.

Technic

Note: If globulin is to be estimated by actual determination on the precipitate as reserved in the albumin analysis (Method A-11, Step 7), omit the following Steps 1–6 inclusive.

1. Place a 15 ml glass-stoppered centrifuge tube in a 150 ml beaker half-filled with warm water at 37° C.
2. Without removing the tube from the beaker, add 7.5 ml 23% sodium sulfate (2), removed from the incubator immediately before use.
3. Add 0.5 ml sample from an Ostwald-Folin pipette, thoroughly rinsing out the pipette by drawing in and expelling some of the mixture several times.
4. Stopper the tube and mix the contents by inversion.
5. Add 4 ml ether (3) and shake vigorously for 30 seconds.
6. Centrifuge the unstoppered tube at 2000 rpm for 15 minutes—the precipitated globulin is tightly packed in a layer at the interface between the sulfate solution and the ether.
7. Slant the tube so that the globulin layer is separated from the walls; with a hooked nichrome wire remove the precipitated globulin en masse and transfer to a small dry Whatman #40 filter paper in a dry funnel. RESERVE SULFATE LAYER FOR ALBUMIN ANALYSIS (Method A-11). Note: If the compact layer should disintegrate, remove as much of the ether as possible by means of a capillary pipette and filter the entire contents of the tube through a small dry Whatman #40 paper, reserving a portion of the filtrate for the albumin analysis before proceeding with the next step.
8. Wash the globulin precipitate with three 3 ml portions 23% sodium sulfate (2)—in the case of a disintegrated precipitate, rinse out the centrifuge tube with three 3 ml portions 23% sodium sulfate, pouring the washings over the precipitate on the paper and discarding the washings.
9. Insert the funnel into a test tube and puncture a small hole in the tip of the paper.
10. Wash all the globulin precipitate from the paper into the tube by means of small portions 0.85% sodium chloride (4) delivered forcibly in a very fine stream from a capillary-tipped wash bottle.
11. Open the filter paper and make certain that all the globulin has been washed into the tube—the total volume in the tube should not exceed 15 ml.
12. Add 0.4 ml 2.5 N sodium hydroxide (5).
13. Heat in water bath until globulin precipitate has dissolved.
14. Cool in running tap water for 3 minutes.
15. Quantitatively transfer the contents of the tube to a 50 ml volumetric flask, repeatedly washing out the tube with small portions of ammonia-free distilled water (1), adding the washings to the flask.

16. Add 0.8 ml selenium-sulfuric acid digestion mixture "C" (6), dilute to volume with ammonia-free distilled water (1) and mix thoroughly.
17. In a special dry digestion tube (sample) place
    0.5 ml diluted globulin solution from Step 16
    1 ml selenium-sulfuric acid digestion mixture "C" (6)
    1 solid glass bead.
18. Clamp tube in a vertical position on a support and apply gentle heat from an Argand micro burner in such a manner that the flame at no time touches the tube above the level of the liquid.

Note: Carry out Steps 18–24 inclusive in a fume hood.

19. After boiling has started gradually increase the heat and place burner in upright position so that the top is about 3/4 inch below the bottom of the tube and the point of the flame directly under the tube—the bubbles should at no time rise above the middle of the tube.
20. Heat until white fumes of SO_3 fill about one-half of the tube—none of these fumes should escape.
21. Insert a small funnel into the mouth of the tube and continue gentle heating with reduced flame, until the charred digest begins to clear.
22. Remove the flame and allow to cool for 90 seconds.
23. Remove the funnel and allow 1 drop 30% hydrogen peroxide (7) to fall directly on the digest.
24. Replace funnel and heat gently for 2 minutes making certain that any charred material which may have adhered to the walls of the tube has been washed down and decomposed—the digest should be perfectly clear—if not, add another drop of hydrogen peroxide in the same manner and heat again until clear.
25. Without removing the funnel, allow to cool for 2 minutes and then place the tube in a beaker half-full of ice-cold water.
26. In another special digestion tube (reagent blank) place 1 ml selenium-sulfuric acid digestion mixture "C" (6) and place in the cold water bath with the other tube.
27. After 10 minutes in the cold water bath, add about 8 ml ammonia-free distilled water (1) to each tube, rinsing off the funnel in the sample tube at the same time and mix, allowing the tubes to remain in the bath.
28. Without removing the tubes from the bath add 10 ml Nessler reagent (8), allowing the reagent to fall directly into the solution.
29. Remove the tubes from the bath, dilute to 20 ml with ammonia-free distilled water (1), insert the glass stoppers and mix by inversion.
30. Replace the tubes in the bath and allow to remain for 5 minutes.
31. Remove the tubes from the bath and allow to stand at room temperature (21° C.) for 10 minutes.
32. Measure the transmittancy of the sample against the reagent blank set at 100% transmission at a wave length of **415** mμ and obtain the concentration of globulin nitrogen from the calibration table.

Calculation

To determine the globulin concentration—
Globulin nitrogen in mg per 100 ml × 0.00625 =
Concentration of globulin in per cent

CALIBRATION PROCEDURE

Special Apparatus

Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.
Fister digestion tubes, glass-stoppered as described in the Test Procedure.
Micro burner, Argand type.

Reagents

1. **Water, Distilled, Ammonia-Free.** Same as for Test Procedure.
6. **Selenium-Sulfuric Acid Digestion Mixture "C" (Fister).** Same as for Test Procedure.
7. **Hydrogen Peroxide, 30%.** Same as for Test Procedure.
8. **Nessler Reagent (Folin-Wu).** Same as for Test Procedure.
9. **Globulin Standards**
    (a) Nitrogen stock standard (1 ml contains 10 mg nitrogen). Dry pyridine-free ammonium sulfate ($(NH_4)_2SO_4$—Reagent Chemicals, Special) at 110° C. for 1 hour; transfer 4.7166 gm to a 100 ml volumetric flask; add approximately 50 ml ammonia-free distilled water (1); shake until dissolved and dilute to volume with same. Preserve with a few drops of toluene. Stability indefinite.
    (b) Dilute standard (1 ml contains 0.1 mg nitrogen). In a 100 ml volumetric flask place 1 ml stock standard (9a) and dilute to volume with ammonia-free distilled water (1). Prepare just before use.
    (c) Calibration standards. In a series of six accurately calibrated 10 ml volumetric flasks place respectively 0, 1, 2, 3, 4 and 5 ml of the dilute standard (9b) and dilute to volume with ammonia-free distilled water (1). These standards represent concentrations of nitrogen equivalent to 0, 200, 400, 600, 800 and 1000 mg per 100 ml sample. Prepare just before use.

Technic

1. Into correspondingly marked special dry digestion tubes place
    1 ml of each calibration standard (9c)
    1 ml selenium-sulfuric acid digestion mixture "C" (6)
    1 solid glass bead.

2. Treat each tube exactly as outlined in the Test Procedure, Steps 18–31 inclusive—the standards are run consecutively through Steps 18–25 inclusive, Step 26 is omitted (since this duplicates the zero concentration calibration standard) and then simultaneously through Steps 27–31 inclusive.
3. Set the zero concentration standard at 100% transmission at a wave length of **415** mμ and record the transmittancy of the 200, 400, 600, 800 and 1000 mg per cent standards. The transmittancy of the 1000 mg standard should be approximately 23.5%.
4. Plot the observed values on semi-log graph paper—the points fall on a very slight curve which passes through the concentration axis slightly to the right of the origin (Calibration Curve G-16).
5. Prepare a calibration table from the T-C values obtained from the plotted curve.

PROCEDURE FOR USE WITH LINEAR-SCALE PHOTOELECTRIC COLORIMETERS (SHEARD-SANFORD TYPE)

TEST PROCEDURE

Exactly as outlined; set the reagent blank at 100 and use a blue filter.

CALIBRATION PROCEDURE

Exactly as outlined—use a blue filter; plot the observed readings on semi-log graph paper—the points fall on a slight curve which passes through the concentration axis slightly to the right of the origin.

PROCEDURE FOR USE WITH LOGARITHMIC-SCALE PHOTOELECTRIC COLORIMETERS (KLETT-SUMMERSON TYPE)

TEST PROCEDURE

Exactly as outlined; set the reagent blank at 0 and use a blue filter.

CALIBRATION PROCEDURE

Exactly as outlined—use a blue filter; plot the observed readings on linear graph paper—the points fall on a slight curve which passes through the concentration axis slightly to the right of the origin.

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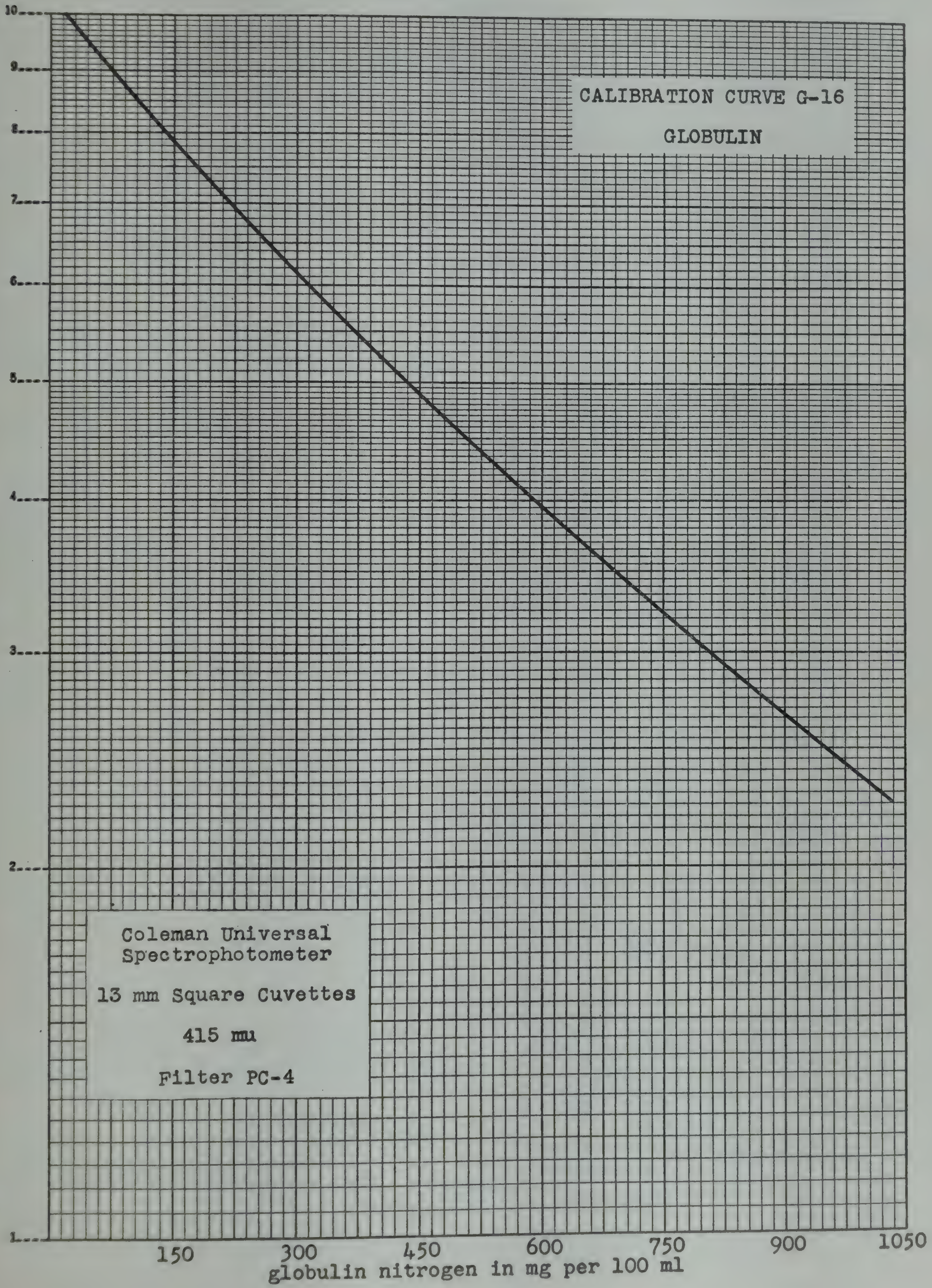
CALIBRATION CURVE G-16
GLOBULIN
Coleman Universal Spectrophotometer
13 mm Square Cuvettes
415 mμ
Filter PC-4
10
9
8
7
6
5
4
3
2
1
150
300
450
600
750
900
1050
globulin nitrogen in mg per 100 ml

Meter Reading	0	1	2	3	4	5	6	7	8	9
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20										
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40										
50										
60										
70										
80										
90										

GLOBULIN

A spectrophotometric adaptation based on the methods and modifications of Howe (1921), Wu (1922), Wu and Ling (1927), Folin and Ciocalteu (1927), Greenberg (1929), Andersch and Gibson (1933), Greenberg and Mirolubova (1936), Kingsley (1940) and Maclay and Osterberg (1940)

Either of two methods may be used for the estimation of globulin—by difference or by actual determination.

A. By Difference

1. Determine the concentration of albumin as outlined in Method A-12.
2. Determine the concentration of fibrinogen as outlined in Method F-12 and divide the value in mg per 100 ml by 1000 to convert to per cent.
3. Determine the concentration of total protein as outlined in Method P-52.
4. Calculation—
   Total protein in % − (albumin in % + fibrinogen in %) = globulin in %

Note: The determination of fibrinogen in routine practice is usually omitted and not corrected for in the calculation.

B. By Determination

PRINCIPLE

Globulin is precipitated by sodium sulfate, the density of which is decreased by the use of ether so that the precipitated globulin forms in a compact mass at the interface between the two layers. The globulin is dissolved in sodium chloride and determined by means of the Folin-Ciocalteu phenol reagent as outlined in the method for total protein (Method P-52), the same analytical principles being involved. One milligram tyrosine produces a color equivalent to the color produced by 11.5 mg globulin.

DETERMINATION IN PLASMA OR SERUM

SAMPLE

Plasma or serum from blood withdrawn during postabsorptive state. Anticoagulant—potassium oxalate. There must be no trace of hemolysis. Plasma or serum must be separated from the red blood cells as soon as possible.

TEST PROCEDURE

Special Apparatus

Glass-stoppered centrifuge tubes, 15 ml.
Ostwald-Folin pipettes accurately calibrated "to contain" 0.5 ml.
Hooked nichrome wire embedded in a glass rod.
Graduated test tubes—25 ml in 0.5 ml; the 20 ml graduation must be accurately calibrated.
Ostwald-Folin pipettes accurately calibrated "to deliver" 5 ml.
Wash bottle, 125 ml with capillary tip.
Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.

Reagents

1. **Sodium Sulfate, 23% Aqueous.** Heat approximately 200 ml distilled water to 37° C.; half fill a 100 ml volumetric flask with the warm water; add 23 gm anhydrous sodium sulfate (Na_2SO_4—Baker & Adamson, Reagent Special); add more warm water until the body of the flask is just about filled; stopper and shake until solution is complete; dilute to volume with the warm water. Keep in incubator at 37° C.

Note: Reagent grade sodium sulfate may vary considerably in its free acid or free alkali content. Each new lot should be tested as follows: dissolve 5 gm in 60 ml carbon dioxide-free distilled water and add 3 drops phenolphthalein indicator solution—no pink color should appear; add 0.5 ml 0.02 N sodium hydroxide—a pink color should appear. Sodium sulfate which does not show these changes is unsatisfactory for the differential precipitation of proteins and should be discarded—the reaction of the final solution must be just on the acid side of neutrality.

2. **Ether, Anhydrous** ($(C_2H_5)_2O$—Mallinckrodt, A.C.S.).
3. **Sodium Chloride, 0.85% Aqueous.** Dissolve 8.5 gm sodium chloride (NaCl—Merck, Special for Biological Work) in distilled water and dilute to 1000 ml.
4. **Sodium Hydroxide, Volumetric 2.5 N.** Prepare by diluting slightly more than the required amount of concentrated carbonate-free sodium hydroxide solution (see Appendix); dilute 20 ml of this approximately 2.5 N solution to 100 ml; titrate against standard 0.5 N acid using phenolphthalein as indicator; adjust accordingly and re-titrate—20 ml of 0.5 N acid require 20 ml of a 1:5 dilution of 2.5 N alkali. Preserve in ceresin-lined bottle.
5. **Phenol Reagent (Folin-Ciocalteu).** In a 1500 ml boiling flask with ground joint place 500 ml

distilled water; add 100 gm sodium tungstate ($Na_2WO_4 \cdot 2H_2O$—Mallinckrodt, Folin Special) and shake until dissolved; add 25 gm sodium molybdate ($Na_2MoO_4 \cdot 2H_2O$—Mallinckrodt, AR); add 200 ml distilled water, washing down the neck of the flask and shake until solution is complete; add 50 ml 85% phosphoric acid (H_3PO_4—Baker & Adamson, A.C.S.) and mix thoroughly; add 100 ml concentrated hydrochloric acid (HCl—Baker & Adamson, A.C.S.) and mix thoroughly; fit a Liebig reflux condenser to the flask and boil gently for 10 hours (during this period the solution changes in color from a pale yellow to a deep green); remove the reflux condenser; add 150 gm lithium sulfate ($LiSO_4$—Baker & Adamson, Reagent) and 50 ml distilled water, washing down the neck of the flask; shake thoroughly until completely dissolved; add 10 drops bromine (Br—Baker & Adamson, A.C.S.) and boil for 15 minutes to remove the excess bromine; allow to cool to room temperature; dilute to 1000 ml with distilled water and filter through tightly packed glass wool into a brown glass-stoppered bottle. The reagent should show no tint of green and if kept well protected from dust will remain stable for months. A reagent which develops a green color in the course of time may be repurified by the addition of a few drops of bromine and boiling as before.

Technic

Note: If globulin is to be estimated by actual determination on the precipitate as reserved in the albumin analysis (Method A-12, Step 7), omit the following Steps 1–6 inclusive.

1. Place a 15 ml glass-stoppered centrifuge tube in a 150 ml beaker half-filled with warm water at 37° C.
2. Without removing the tube from the beaker, add 7.5 ml 23% sodium sulfate (1), removed from the incubator immediately before use.
3. Add 0.5 ml sample from an Ostwald-Folin pipette, thoroughly rinsing out the pipette by drawing in and expelling some of the mixture several times.
4. Stopper the tube and mix the contents by inversion.
5. Add 4 ml ether (2) and shake vigorously for 30 seconds.
6. Centrifuge the unstoppered tube at 2000 rpm for 15 minutes—the precipitated globulin is tightly packed in a layer at the interface between the sulfate solution and the ether.
7. Slant the tube so that the globulin layer is separated from the walls; with a hooked nichrome wire remove the precipitated globulin en masse and transfer to a small dry Whatman #40 filter paper in a dry funnel. RESERVE SULFATE LAYER FOR ALBUMIN ANALYSIS (Method A-12). Note: If the compact layer should disintegrate, remove as much of the ether as possible by means of a capillary pipette and filter the entire contents of the tube through a small dry Whatman #40 paper, reserving a portion of the filtrate for the albumin analysis before proceeding with the next step.
8. Wash the globulin precipitate with three 3 ml portions 23% sodium sulfate (1)—in the case of a disintegrated precipitate, rinse out the centrifuge tube with three 3 ml portions 23% sodium sulfate, pouring the washings over the precipitate on the paper and discarding the washings.
9. Insert the funnel into a 25 ml graduated test tube and puncture a small hole in the tip of the paper.
10. Wash all the globulin precipitate from the paper into the tube by means of small portions 0.85% sodium chloride (3) delivered forcibly in a very fine stream from a capillary-tipped wash bottle.
11. Open the filter paper and make certain that all the globulin has been washed into the tube—the total volume in the tube should not exceed 5 ml.
12. Add 4 ml 2.5 N sodium hydroxide (4)
    1 ml distilled water.
13. Place in boiling water for 10 minutes.
14. Cool in running tap water for 3 minutes.
15. Dilute to 20 ml with distilled water, mix thoroughly by inversion and transfer 5 ml to a 100 ml volumetric flask (sample), using an Ostwald-Folin pipette calibrated "to deliver."
16. Add 3 ml 2.5 N sodium hydroxide (4).
17. In another 100 ml volumetric flask (reagent blank) place
    4 ml 2.5 N sodium hydroxide (4).
18. To each flask add about 50 ml distilled water and mix.
19. Add 3 ml phenol reagent (5), slowly and with constant agitation of the flask.
20. Dilute to volume with distilled water and mix thoroughly by inversion.
21. Allow to stand at room temperature for 10 minutes.
22. Measure the transmittancy of the sample against the reagent blank set at 100% transmission at a wave length of 700 mμ and multiply the value from the calibration table for total protein (Method P-52) by 0.4 to obtain the concentration of globulin in per cent. If a calibration table has been prepared for globulin, the concentration is obtained directly.

CALIBRATION PROCEDURE

Note: The following calibration procedure is optional. The values in the calibration table for total protein (Method P-52), when multiplied by 0.4, are equivalent to the concentration of globulin in per cent.

Special Apparatus

Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.

Reagents

4. **Sodium Hydroxide, Volumetric 2.5 N.** Same as for Test Procedure.
5. **Phenol Reagent (Folin-Ciocalteu).** Same as for Test Procedure.
6. **Hydrochloric Acid, Volumetric 0.1 N.** Add 10 ml concentrated hydrochloric acid (HCl—Baker & Adamson, A.C.S.) to 1000 ml distilled water and mix; standardize against 0.1 N alkali using phenolphthalein as indicator.
7. **Globulin Standards**
    (a) Tyrosine stock standard (1 ml contains 0.2 mg tyrosine). In a 100 ml volumetric flask place 20 mg l-tyrosine ($C_9H_{11}O_3N$—Pfanstiehl, C.P.), add about 85 ml 0.1 N hydrochloric acid (6); shake until dissolved and dilute to volume with same. Preserve with a few drops of xylene.
    (b) Dilute standard (1 ml contains 0.05 mg tyrosine). In a 100 ml volumetric flask place 25 ml stock standard (7a) and dilute to volume with distilled water. Prepare just before use.
    (c) Calibration standards. In a series of six accurately calibrated 100 ml volumetric flasks place respectively 0, 2.5, 5, 7.5, 10 and 12.5 ml of the dilute standard (7b) and proceed as follows. These standards represent concentrations of globulin equivalent to 0, 1.15, 2.30, 3.45, 4.60 and 5.75%. Prepare just before use.

Technic

1. To each calibration standard (7c) add 4 ml 2.5 N sodium hydroxide (4).
2. Proceed exactly as outlined in the Test Procedure, Steps 18–21 inclusive.
3. Set the zero concentration standard at 100% transmission at a wave length of **700 $m\mu$** and record the transmittancy of the 1.15, 2.30, 3.45, 4.60 and 5.75% standards. The transmittancy of the 5.75% standard should be approximately 27%.
4. Plot the observed values on semi-log graph paper—the points fall on a straight line which passes through the origin (Calibration Curve G-17).
5. Prepare a calibration table from the T-C values obtained from the plotted curve.

PROCEDURE FOR USE WITH LINEAR-SCALE PHOTOELECTRIC COLORIMETERS (SHEARD-SANFORD TYPE)

TEST PROCEDURE

Exactly as outlined; set the reagent blank at 100 and use a red filter.

CALIBRATION PROCEDURE

Exactly as outlined—use a red filter; plot the observed readings on semi-log graph paper—the points fall on a straight line passing through the origin.

PROCEDURE FOR USE WITH LOGARITHMIC-SCALE PHOTOELECTRIC COLORIMETERS (KLETT-SUMMERSON TYPE)

TEST PROCEDURE

Exactly as outlined; set the reagent blank at 0 and use a red filter.

CALIBRATION PROCEDURE

Exactly as outlined—use a red filter; plot the observed readings on linear graph paper—the points fall on a straight line passing through the origin.

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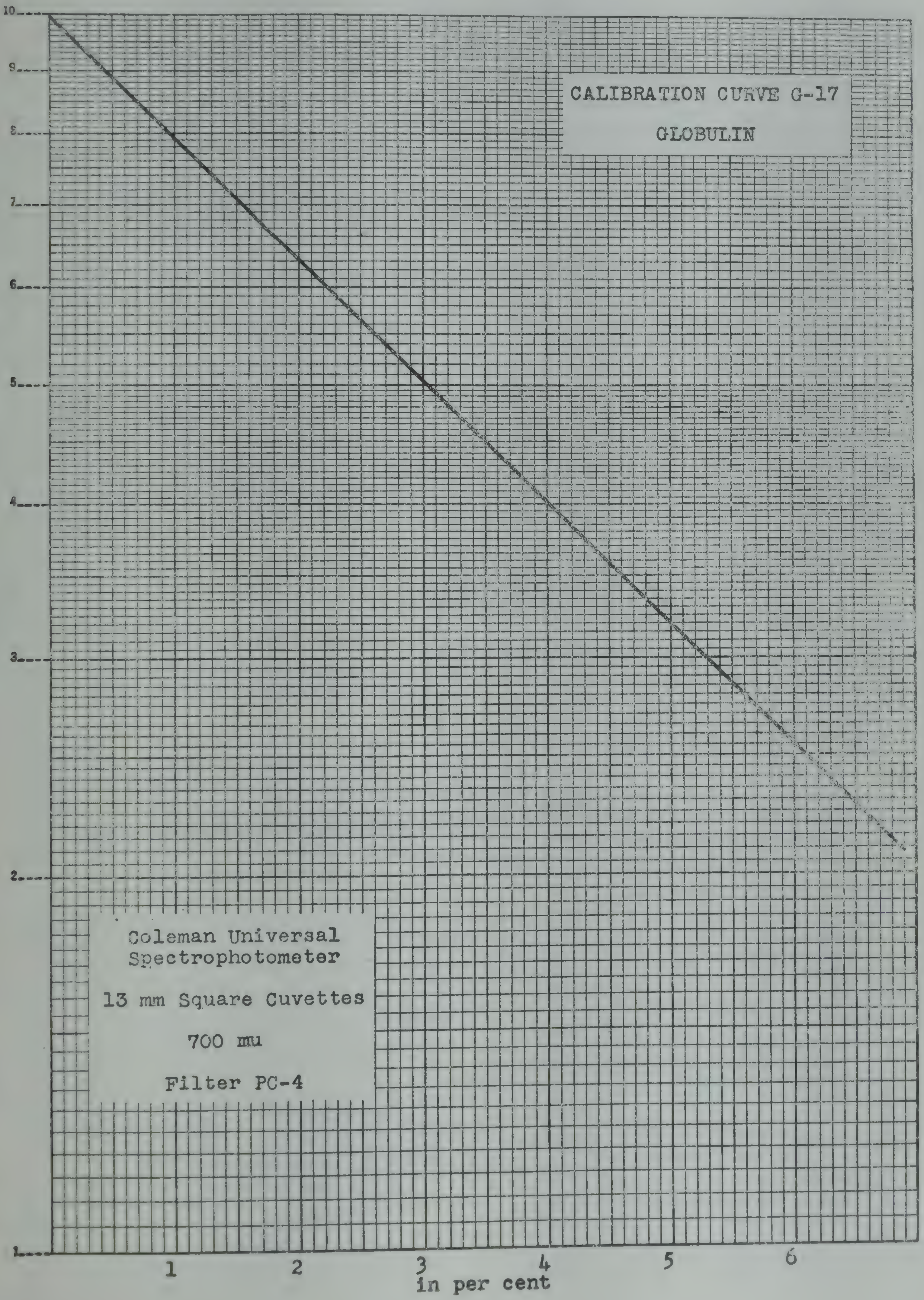
NOTES



CALIBRATION CURVE G-17

GLOBULIN

Coleman Universal Spectrophotometer

13 mm Square Cuvettes

700 mu

Filter PC-4

Meter Reading	0	1	2	3	4	5	6	7	8	9
0										
10										
20										
30										
40										
50										
60										
70										
80										
90										

GUANIDINE

A spectrophotometric adaptation based on the methods and modifications of Weber (1927; 1928), Major and Weber (1927), Pfiffner and Myers (1930) and Andes and Myers (1937)

PRINCIPLE

Guanidine is adsorbed from the blood by charcoal in alkaline solution from which it is released by boiling acid alcohol. Interference by creatine is minimized by hydrolysis to creatinine. Guanidine is determined by the red color developed upon treatment of the extract with alkaline ferricyanide-nitroprusside (Tieg reaction). Correction must be made for the effect of creatine and creatinine. Since this method affords only 82% recovery, a further correction factor is necessary.

DETERMINATION IN WHOLE BLOOD

SAMPLE

Blood withdrawn during post-absorptive state. Analyses should be made as soon as possible after withdrawal of the blood.

TEST PROCEDURE

Special Apparatus

Folin-Wu pipettes, 10 ml; the 8 ml graduation must be accurately calibrated.

Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.

Reagents

1. **Sulfuric Acid, Volumetric N/12.** Add 2.5 ml concentrated sulfuric acid (H_2SO_4—Grasselli, A.C.S.) to 1000 ml distilled water and mix well; titrate against standard 0.1 N alkali using phenolphthalein as indicator; adjust accordingly and retitrate—20 ml of N/12 acid require 16.7 ml of 0.1 N alkali.
2. **Sodium Tungstate, 10% Aqueous.** Dissolve 10 gm sodium tungstate ($Na_2WO_4 \cdot 2H_2O$—Mallinckrodt, Folin Special) in distilled water and dilute to 100 ml. The solution must be neutral or slightly alkaline to phenolphthalein, not more than 0.4 ml being required to neutralize 10 ml 0.1 N hydrochloric acid. Preserve in resistant-glass bottle.
3. **Sodium Hydroxide, Volumetric 2.5 N.** Prepare by diluting slightly more than the required amount of concentrated carbonate-free sodium hydroxide solution (see Appendix); dilute 20 ml of this approximately 2.5 N solution to 100 ml; titrate against standard 0.5 N acid using phenolphthalein as indicator; adjust accordingly and re-titrate—20 ml of 0.5 N acid require 20 ml of a 1:5 dilution of 2.5 N alkali. Preserve in ceresin-lined bottle.
4. **Norit A, Acid-Washed (Andes-Myers).** In a 1000 ml beaker place 100 gm norit A (Pfanstiehl) and add 100 ml 0.5 N hydrochloric acid (a); heat to boiling and filter while hot on a Büchner funnel with suction; wash the norit on the filter with successive portions of distilled water until the washings are chloride-free; remove from filter and dry in a hot air oven at 120° C. for 24 hours—do not heat to high temperatures.
   (a) Hydrochloric acid, volumetric 0.5 N; dilute 5 ml 10 N hydrochloric acid (Reagent #6a) to 100 ml with distilled water and mix.
5. **Alkaline Wash Solution.** Add 6 ml 2.5 N sodium hydroxide (3) to 1000 ml distilled water and mix.
6. **Acid Alcohol Solution.** Dilute 1 ml 10 N hydrochloric acid (a) to 500 ml with 95% ethyl alcohol (Reagent #7).
   (a) Hydrochloric acid, volumetric 10 N; dilute 850 ml concentrated hydrochloric acid (HCl—Baker & Adamson, A.C.S.) to 1000 ml with distilled water; mix well and allow to cool; dilute 10 ml of this approximately 10 N solution to 100 ml; titrate against standard 1 N alkali using phenolphthalein as indicator; adjust accordingly and retitrate—20 ml of a 1:10 dilution of 10 N acid require 20 ml of 1 N alkali. Preserve in glass-stoppered bottle.
7. **Ethyl Alcohol, 95%** (C_2H_5OH—U. S. Industrial Chemicals, U.S.P.).
8. **Hydrochloric Acid, Volumetric 0.2 N.** Dilute 2 ml 10 N hydrochloric acid (6a) to 100 ml with distilled water and mix.
9. **Ethyl Alcohol, Absolute** (C_2H_5OH—U. S. Industrial Chemicals, U.S.P.).
10. **Water, Distilled, Ammonia-Free.** In an all-glass Pyrex distillation apparatus place 1 gm sodium carbonate (Na_2CO_3—Baker & Adamson, A.C.S.) and 1 gm potassium permanganate ($KMnO_4$—Baker & Adamson, A.C.S.); add 500 ml distilled water; re-distil, rejecting the first 100 ml portion and collecting only the next 300 ml.
11. **Ferricyanide-Nitroprusside Reagent (Weber).** In a 250 ml Erlenmeyer flask place 10 ml 2.5 N sodium hydroxide (3), 10 ml 10% sodium nitroprusside (a) and 10 ml 10% potassium ferricyanide (b); mix and add 90 ml ammonia-free distilled water (10); allow to stand for 20 minutes—the deep reddish-brown color changes to a pale yellow and the reagent is then ready for use. Stable for a considerable time if preserved in a brown bottle in the refrigerator. A slight precipitate does not

affect the reagent and may be filtered off, but discard if a marked turbidity develops.

- (a) Sodium nitroprusside, 10% aqueous; dissolve 1 gm crystalline sodium nitroprusside ($Na_2Fe(CN)_5(NO) \cdot 2H_2O$—Baker & Adamson, A.C.S.) in ammonia-free distilled water (10) and dilute to 10 ml. Prepare just before use.
- (b) Potassium ferricyanide, 10% aqueous; dissolve 1 gm crystalline potassium ferricyanide ($K_3Fe(CN)_6$—Baker & Adamson, A.C.S.) in ammonia-free distilled water (10) and dilute to 10 ml. Prepare just before use.

Technic

Note: For normal blood and in the absence of nitrogen retention, use double the amount of sample and double the amount of all the reagents through Step 20; proceed exactly as outlined from Steps 21–26 inclusive and divide the values obtained from the calibration table by 2 before calculating the concentration of methylguanidine base.

1. In a 125 ml Erlenmeyer flask place
   64 ml N/12 sulfuric acid (1).
2. Add 8 ml sample from a Folin-Wu pipette, thoroughly rinsing out the pipette by drawing in and expelling some of the mixture several times.
3. Mix the contents of the flask by gentle rotation until the fluid is uniformly black-brown.
4. Add 8 ml 10% sodium tungstate (2), drop by drop, while constantly, but gently, rotating the flask.
5. Shake the flask vigorously until the mixture is chocolate colored and foam-free.
6. Filter through a dry Whatman #4 paper into a dry flask.
7. Transfer 50 ml filtrate to a 125 ml Erlenmeyer flask—RESERVE THE REMAINDER OF THE FILTRATE FOR THE DETERMINATION OF CREATINE AND CREATININE.
8. Add 5 drops 2.5 N sodium hydroxide (3) and mix.

Note: The procedure from Steps 9–12 inclusive should be carried out as quickly as possible.

9. Add approximately 125 mg (±5 mg) acid-washed norit A (4), shake thoroughly and allow to stand for 2 minutes.
10. Filter, by means of suction, through a Schleicher and Schuell #576 paper, rinsing out the flask with 4 ml alkaline wash solution (5) and pouring the washing onto the filter; suck dry and discard the filtrate.
11. Return the filter paper and norit to the original flask.
12. Immediately add 25 ml acid alcohol solution (6) and shake.
13. Place the flask on a water bath and evaporate to dryness at 80° C.—evaporation may be facilitated by drawing a current of air through the flask.
14. To the dry residue in the flask add 25 ml 95% ethyl alcohol (7), shake and allow to stand for 2 minutes.
15. Filter through a Schleicher and Schuell #576 paper into a dry flask.
16. Transfer 20 ml filtrate to a 50 ml Erlenmeyer flask and again evaporate to dryness as above—do not overheat.
17. Take up the residue in 2 ml 0.2 N hydrochloric acid (8).
18. Cover mouth of flask with tinfoil and autoclave at 120° C. for 20 minutes.
19. Place flask on water bath and again evaporate to dryness as before.
20. Take up the residue in 2 ml absolute ethyl alcohol (9) and again evaporate to dryness as before.
21. Add 5 ml ammonia-free distilled water (10) and swirl gently.
22. Add 1.5 ml ferricyanide-nitroprusside reagent (11) and mix gently.
23. Transfer to a 15 ml centrifuge tube and centrifuge at 2000 rpm for 3 minutes to remove precipitate which always forms at this point.
24. Carefully decant the clear supernatant fluid into a cuvette (sample).
25. In another cuvette (reagent blank) place
    5 ml ammonia-free distilled water (10)
    1.5 ml ferricyanide-nitroprusside reagent (11).
26. Immediately (or not longer than *8* minutes after the addition of the ferricyanide-nitroprusside reagent) measure the transmittancy of the sample against the reagent blank set at 100% transmission at a wave length of **510** mμ; obtain the value from the calibration table and calculate the concentration of methylguanidine base as follows.

Calculation

To correct for creatine and creatinine concentration in sample—

Determine the concentration of creatine and creatinine in the protein-free filtrate reserved in Step 7 by any of the outlined methods and apply the following formula:

Value for methylguanidine base from calibration table in mg per 100 ml − ([creatinine in mg per 100 ml × 0.014] + [creatine in mg per 100 ml × 0.01]) = apparent concentration of methylguanidine base in mg per 100 ml

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To correct for incomplete recovery multiply the apparent concentration obtained above by 1.22 to obtain the true concentration of methylguanidine base in mg per 100 ml.

CALIBRATION PROCEDURE

Special Apparatus

Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.

Graduated test tubes—10 ml in 0.1 ml; the 5 ml graduation must be accurately calibrated.

Reagents

10. **Water, Distilled, Ammonia-Free.** Same as for Test Procedure.
11. **Ferricyanide-Nitroprusside Reagent (Weber).** Same as for Test Procedure.
12. **Guanidine Standards**
    (a) Guanidine stock standard (1 ml contains 1 mg methylguanidine base). In a 100 ml volumetric flask place 167.1 mg methylguanidine sulfate ($(C_2H_7N_3)_2 \cdot H_2SO_4$—Eastman #1482); add approximately 50 ml ammonia-free distilled water (10) and shake until dissolved; dilute to volume with same. Preserve in refrigerator.
    (b) Dilute standard (1 ml contains 0.02 mg methylguanidine base). In a 100 ml volumetric flask place 2 ml stock standard (12a) and dilute to volume with ammonia-free distilled water (10). Prepare just before use.
    (c) Calibration standards. In a series of six accurately calibrated 10 ml test tubes place respectively 0, 1, 2, 3, 4 and 5 ml of the dilute standard (12b); add 5, 4, 3, 2, 1 and 0 ml ammonia-free distilled water (10) respectively, bringing the volume in each tube to 5 ml. These standards represent concentrations of methylguanidine base equivalent to 0, 0.5, 1.0, 1.5, 2.0 and 2.5 mg per 100 ml sample. Prepare just before use and proceed immediately as follows.

Technic

1. To each calibration standard (12c) add 1.5 ml ferricyanide-nitroprusside reagent (11) and mix.
2. Immediately (or not longer than *8* minutes after the addition of the ferricyanide-nitroprusside reagent) set the zero concentration standard at 100% transmission at a wave length of **510 mμ** and record the transmittancy of the 0.5, 1.0, 1.5, 2.0 and 2.5 mg per cent standards. The transmittancy of the 2.5 mg standard should be approximately 43.5%.
3. Plot the observed values on semi-log graph paper —the points fall on a straight line which passes through the origin (Calibration Curve G-20A).
4. Prepare a calibration table from the T-C values obtained from the plotted curve.

Method G-20b

DETERMINATION IN URINE

PRINCIPLE

Substances which interfere are removed from the urine by means of lead acetate and excess lead removed as the phosphate. The analysis is then carried out as outlined for blood, due correction being made for the creatine and creatinine content; however, excessive amounts of creatine invalidate the results. A further correction factor for incomplete recovery is necessary.

SAMPLE

Aliquot of a 24-hour specimen. Keep cool during collection.

TEST PROCEDURE

Special Apparatus

As listed under the Test Procedure for blood.

Reagents

3. **Sodium Hydroxide, Volumetric 2.5 N.** Same as for blood.
4. **Norit A, Acid-Washed (Andes-Myers).** Same as for blood.
5. **Alkaline Wash Solution.** Same as for blood.
6. **Acid Alcohol Solution.** Same as for blood.
7. **Ethyl Alcohol, 95%.** Same as for blood.
10. **Water, Distilled, Ammonia-Free.** Same as for blood.
11. **Ferricyanide-Nitroprusside Reagent (Weber).** Same as for blood.
13. **Lead Acetate, 40% Aqueous.** Dissolve 40 gm crystalline lead acetate ($Pb(C_2H_3O_2)_2 \cdot 3H_2O$—Baker & Adamson, A.C.S.) in distilled water and dilute to 100 ml.
14. **Sodium Phosphate, Dibasic, Saturated Aqueous.** In a four ounce bottle place 7 gm crystalline disodium phosphate ($Na_2HPO_4 \cdot 12H_2O$—Merck, A.C.S.) and add 100 ml distilled water; shake thoroughly at intervals until saturation is complete; use clear supernatant fluid.

Technic

Note: For concentrated specimens of urine, dilute with distilled water so that the creatinine content is less than 100 mg per 100 ml and correct for the dilution accordingly.

1. In a 50 ml centrifuge tube place
   25 ml thoroughly mixed urine
   10 ml 40% lead acetate (13)
   5 ml 2.5 N sodium hydroxide (3).
2. Mix thoroughly and centrifuge at 1500 rpm for 10 minutes.

3. Transfer 30 ml clear supernatant centrifugate (do not filter) to another 50 ml centrifuge tube and add 15 ml saturated disodium phosphate (14)—RESERVE THE REMAINDER OF THE CENTRIFUGATE FOR THE DETERMINATION OF CREATINE AND CREATININE.
4. Mix thoroughly and centrifuge at 1500 rpm for 10 minutes.
5. In a 125 ml Erlenmeyer flask place
   8 ml clear supernatant centrifugate.

Note: All details of the procedure from Steps 6–9 inclusive must be rigidly followed as quickly as possible.

6. Add approximately 200 mg (± 5 mg) acid-washed norit A (4), and shake thoroughly for *exactly* 20 seconds.
7. Filter, by means of suction, through a Schleicher and Schuell #576 paper, rinsing out the flask with two 10 ml portions of alkaline wash solution (5) and pouring the washings onto the filter; suck dry and discard the filtrate.
8. Return the filter paper and norit to the original flask.
9. Immediately add 25 ml acid alcohol solution (6) and shake.
10. Place the flask on a water bath and evaporate to dryness at 80° C.—evaporation may be facilitated by drawing a current of air through the flask.
11. To the dry residue in the flask add 30 ml 95% ethyl alcohol (7), shake thoroughly and filter through a 9 cm Schleicher and Schuell #576 paper into a dry flask.
12. Transfer 25 ml filtrate to a 50 ml Erlenmeyer flask and again evaporate to dryness as above—do not overheat.
13. Take up the residue in 5 ml ammonia-free distilled water (10) and swirl gently.
14. Add 1.5 ml ferricyanide-nitroprusside reagent (11) and mix gently.
15. Transfer to a 15 ml centrifuge tube and centrifuge at 2000 rpm for 3 minutes to remove precipitate which always forms at this point.
16. Carefully decant the clear supernatant fluid into a cuvette (sample).
17. In another cuvette (reagent blank) place
    5 ml ammonia-free distilled water (10)
    1.5 ml ferricyanide-nitroprusside reagent (11).
18. Immediately (or not longer than *8* minutes after the addition of the ferricyanide-nitroprusside reagent) measure the transmittancy of the sample against the reagent blank set at 100% transmission at a wave length of **510** mμ; multiply the value from the calibration table for blood by 1.44 and calculate the concentration of methylguanidine base as follows.

Calculation

To correct for creatine and creatinine concentration in sample—

Determine the concentration of creatine and creatinine in the lead filtrate reserved in Step 3 by any of the outlined methods. Obtain the correction factor for the creatinine concentration from Chart G-20.

Apply the following formula:

Value for methylguanidine base from calibration table in mg per 100 ml − ([creatinine in mg per 100 ml × factor from Chart G-20] + [creatine in mg per 100 ml × 0.031]) = apparent concentration of methylguanidine base in mg per 100 ml

To correct for incomplete recovery multiply the apparent concentration obtained above by 1.14 to obtain the true concentration of methylguanidine base in mg per 100 ml.

CALIBRATION PROCEDURE

When numerous guanidine determinations on urine are required, it is convenient to prepare a calibration curve (Calibration Curve G-20B) and table by calculation from the curve constructed for blood. In this case, the values are obtained directly.

◆

PROCEDURE FOR USE WITH LINEAR-SCALE PHOTOELECTRIC COLORIMETERS (SHEARD-SANFORD TYPE)

TEST PROCEDURE

Exactly as outlined; set the reagent blank at 100 and use a filter with maximum transmission at 510 mμ.

CALIBRATION PROCEDURE

Exactly as outlined—use a filter with maximum transmission at **510** mμ; plot the observed readings on semilog graph paper—the points fall on a straight line passing through the origin.

◆

PROCEDURE FOR USE WITH LOGARITHMIC-SCALE PHOTOELECTRIC COLORIMETERS (KLETT-SUMMERSON TYPE)

TEST PROCEDURE

Exactly as outlined; set the reagent blank at 0 and use a filter with maximum transmission at 510 mμ.

CALIBRATION PROCEDURE

Exactly as outlined—use a filter with maximum transmission at **510** mμ; plot the observed readings on linear graph paper—the points fall on a straight line passing through the origin.

Note: For instruments with cells, cuvettes or tubes requiring a final volume of more than 6.5 ml, add 2 ml ammonia-free distilled water before measurements are made in both the test and calibration procedures.

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Major, R. H., and Weber, C. J., "Possible Increase of Guanidine in the Blood of Certain Persons with Hypertension," *Arch. Int. Med.*, **40,** 891 (1927).

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Andes, J. E., and Myers, V. C., "Guanidine-Like Substances in Blood. I. Colorimetric Estimation and Normal Values," *J. Lab. Clin. Med.*, **22,** 1147 (1937).

Andes, J. E., and Myers, V. C., "Colorimetric Estimation of Guanidine-Like Substances in the Urine," *J. Biol. Chem.*, **118,** 137 (1937).

NOTES

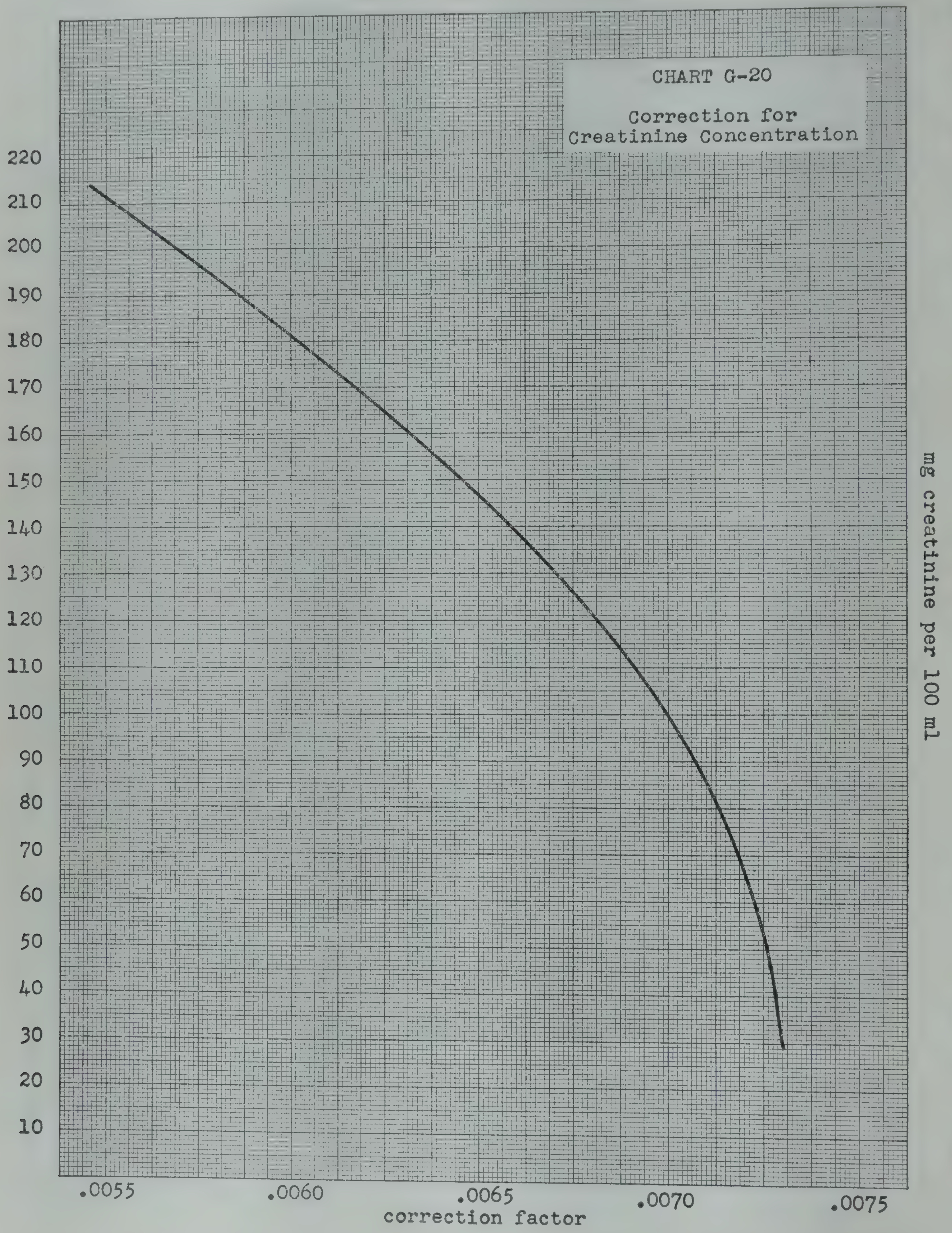
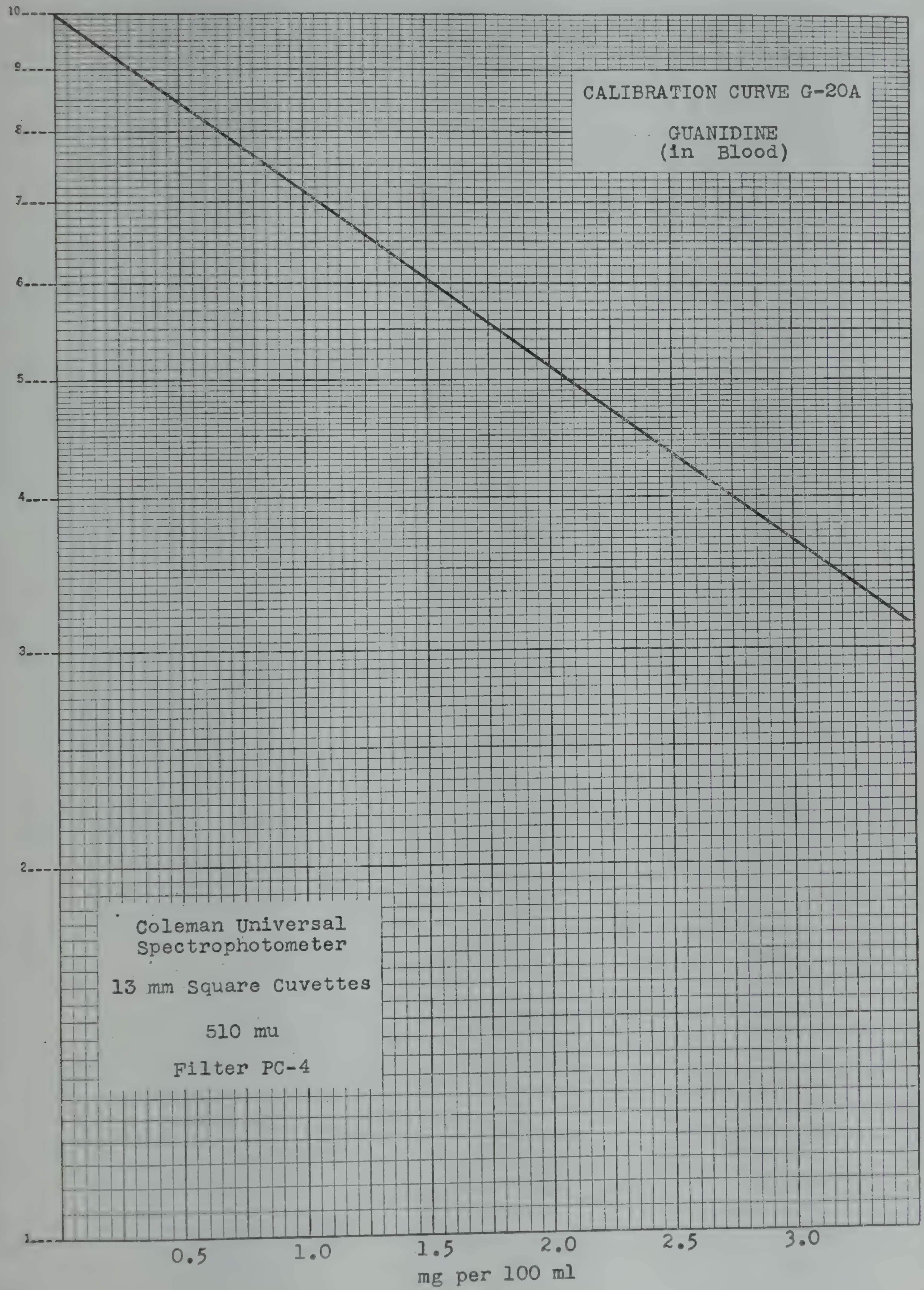
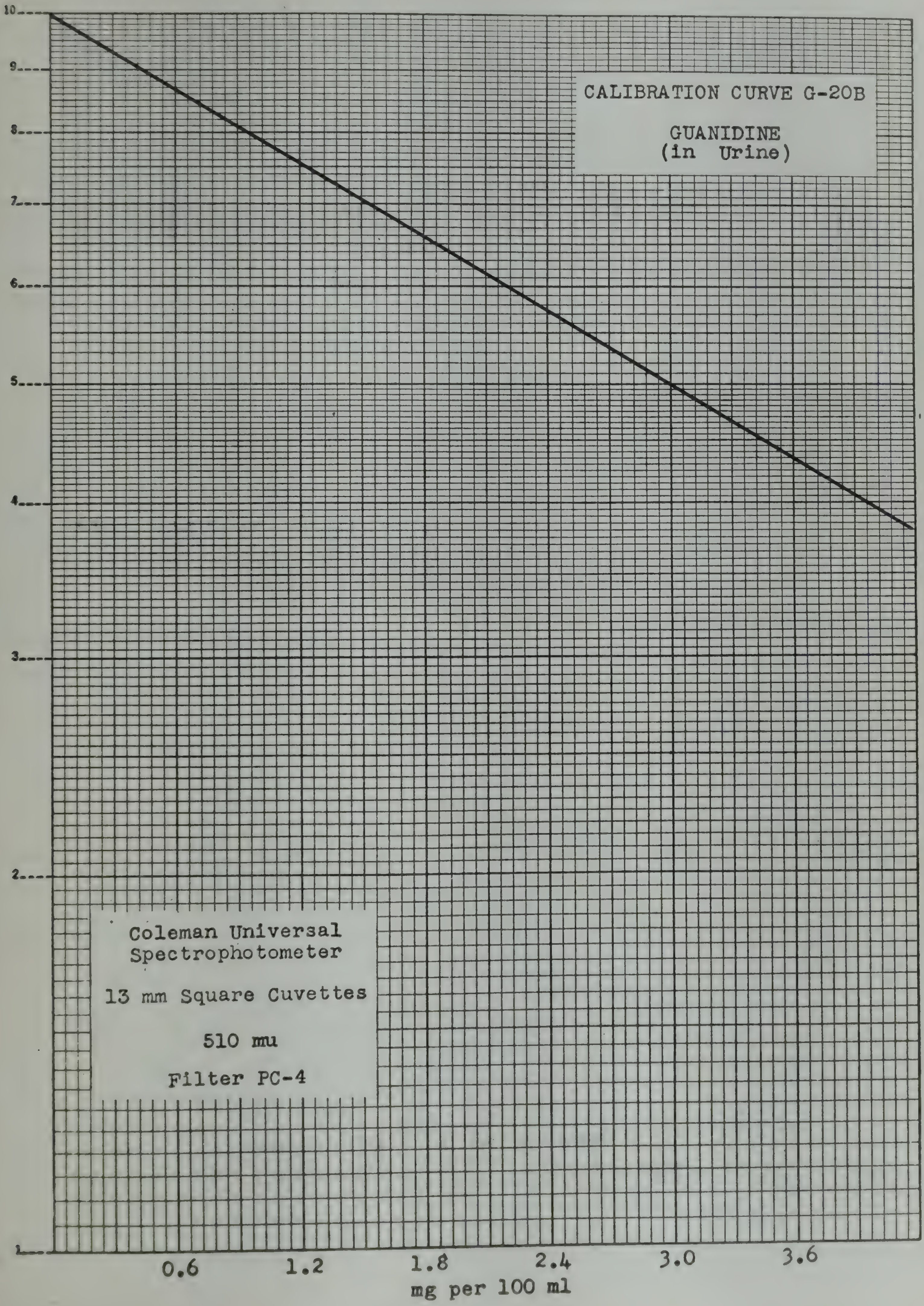


CHART G-20
Correction for
Creatinine Concentration
220
210
200
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
.0055
.0060
.0065
.0070
.0075
correction factor
mg creatinine per 100 ml


CALIBRATION CURVE G-20A
GUANIDINE
(in Blood)
Coleman Universal
Spectrophotometer
13 mm Square Cuvettes
510 mu
Filter PC-4
10
9
8
7
6
5
4
3
2
1
0.5
1.0
1.5
2.0
2.5
3.0
mg per 100 ml

Meter Reading	0	1	2	3	4	5	6	7	8	9
0										
10										
20										
30										
40										
50										
60										
70										
80										
90										


CALIBRATION CURVE G-20B
GUANIDINE
(in Urine)
Coleman Universal
Spectrophotometer
13 mm Square Cuvettes
510 mu
Filter PC-4
10
9
8
7
6
5
4
3
2
1
0.6
1.2
1.8
2.4
3.0
3.6
mg per 100 ml

Meter Reading	0	1	2	3	4	5	6	7	8	9
0										
10										
20										
30										
40										
50										
60										
70										
80										
90										

HEMOGLOBIN (ACID HEMATIN)

A spectrophotometric adaptation based on the method of Sahli (1905)

PRINCIPLE

Hemoglobin is determined by the brownish color of acid hematin produced upon treatment of the blood with hydrochloric acid.

DETERMINATION IN WHOLE BLOOD

SAMPLE

Whole blood—either fresh or oxalated samples may be used. This method should be employed only in the absence of abnormal hemoglobin derivatives; if the presence of such derivatives is suspected, Method H-15 should be used.

TEST PROCEDURE

Special Apparatus

Micro pipettes accurately calibrated "to contain" 25 cmm.

Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.

Reagent

1. **Hydrochloric Acid, Volumetric 0.1 N.** Add 10 ml concentrated hydrochloric acid (HCl—Baker & Adamson, A.C.S.) to 1000 ml distilled water and mix; titrate against standard 0.1 N alkali using phenolphthalein as indicator; adjust accordingly and re-titrate—20 ml of 0.1 N acid require 20 ml of 0.1 N alkali.

Technic

1. In a 25 ml volumetric flask (sample) place 20 ml 0.1 N hydrochloric acid (1).
2. Add 25 cmm fresh or oxalated blood from a micro pipette, thoroughly rinsing out the pipette by drawing in and expelling some of the mixture several times.
3. Dilute to volume with 0.1 N hydrochloric acid (1) and mix thoroughly.
4. Allow to stand at room temperature for 40 minutes.
5. Measure the transmittancy of the sample against 0.1 N hydrochloric acid (1) set at 100% transmission at a wave length of **400** mμ and obtain the concentration of hemoglobin from the calibration table.

CALIBRATION PROCEDURE

Special Apparatus

Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.

Micro pipettes accurately calibrated "to contain" 0.1 ml.

Reagents

1. **Hydrochloric Acid, Volumetric 0.1 N.** Same as for Test Procedure.
2. **Hemoglobin Standards**
   (a) Hemoglobin stock standard. Pool several samples of oxalated blood; centrifuge about 10 ml of the pooled bloods at low speed for 5 minutes; remove and discard about 1 ml of the supernatant plasma and thoroughly mix the remaining plasma and red cells. Determine the total iron content of the concentrated blood and calculate the hemoglobin equivalent as outlined in the methods for total iron (Methods I-27 or I-28). For highest accuracy the average result of several determinations should be used.
   (b) Dilute standard. In a 100 ml volumetric flask place approximately 80 ml 0.1 N hydrochloric acid (1); add 0.1 ml concentrated blood from a micro pipette, thoroughly rinsing out the pipette by drawing in and expelling some of the mixture several times; dilute to volume with 0.1 N hydrochloric acid and mix thoroughly; allow to stand at room temperature for 40 minutes. Prepare just before use and proceed immediately as follows.

Technic

1. Transfer respectively 2.5, 5.0, 7.5 and 10 ml of the dilute standard (2b) to correspondingly marked accurately calibrated 10 ml volumetric flasks (calibration standards).
2. Dilute to volume with 0.1 N hydrochloric acid (1) and mix thoroughly.
3. Immediately set 0.1 N hydrochloric acid (1) at 100% transmission at a wave length of **400** mμ and record the transmittancy of the calibration standards.
4. Calculation of the hemoglobin equivalents of the calibration standards—

   Let Hb represent the concentration of hemoglobin in gm per 100 ml in the stock standard (2a)

   Let V represent the volume of the dilute standard (2b) taken for calibration

   Then

   $$\frac{Hb \times V}{10} = \text{Concentration of hemoglobin in the calibration standard}$$

   Example:

   Iron content of stock standard (2a) = 51 mg per 100 ml

Or, $\frac{51}{3.35}$ = 15.2 gm hemoglobin per 100 ml stock standard (2a)

Therefore, the dilute standard (2b) has a hemoglobin equivalent of 15.2 gm per 100 ml

For the calibration standard containing 2.5 ml of the dilute standard, the calculation is—

$\frac{15.2 \times 2.5}{10}$ or, 3.8 gm hemoglobin per 100 ml blood

The calculation for the other calibration standards is similar and in this case, would be 7.6, 11.4 and 15.2 gm per 100 ml.

5. Plot the calculated hemoglobin equivalents of the calibration standards on semi-log graph paper —the points fall on a slight curve which passes through the origin (Calibration Curve H-10). A hemoglobin concentration of 15.6 gm per 100 ml should show a transmittancy reading of approximately 36%.
6. Prepare a calibration table from the T-C values obtained from the plotted curve.

PROCEDURE FOR USE WITH LINEAR-SCALE PHOTOELECTRIC COLORIMETERS (SHEARD-SANFORD TYPE)

TEST PROCEDURE

Exactly as outlined; set 0.1 N hydrochloric acid at 100 and use a blue filter.

CALIBRATION PROCEDURE

Exactly as outlined—use a blue filter; plot the observed readings on semi-log graph paper—the points fall on a slight curve passing through the origin.

PROCEDURE FOR USE WITH LOGARITHMIC-SCALE PHOTOELECTRIC COLORIMETERS (KLETT-SUMMERSON TYPE)

TEST PROCEDURE

Exactly as outlined; set 0.1 N hydrochloric acid at 0 and use a green filter.

CALIBRATION PROCEDURE

Exactly as outlined—use a green filter; plot the observed readings on linear graph paper—the points fall on a very slight curve passing through the origin.

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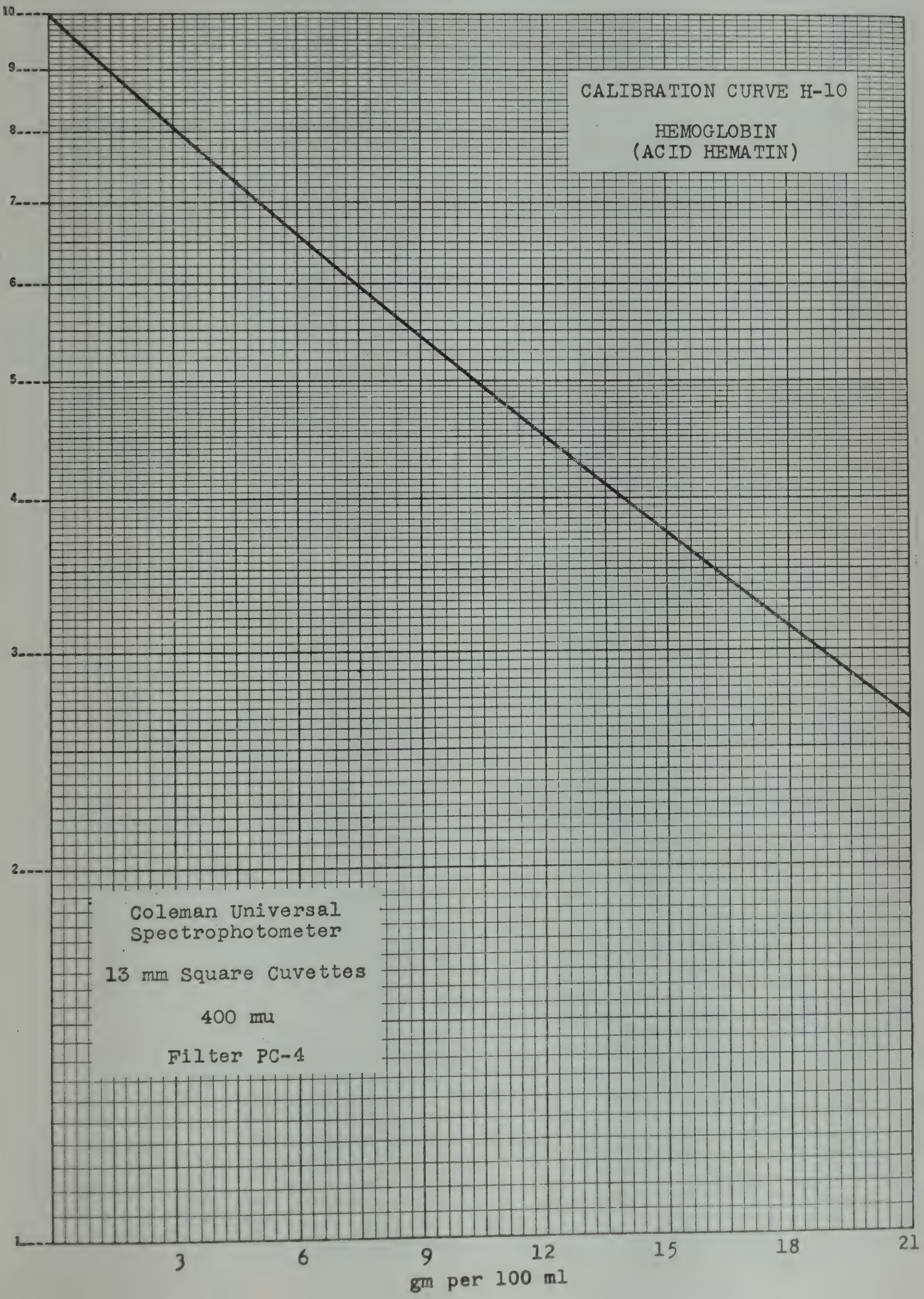
NOTES


CALIBRATION CURVE H-10
HEMOGLOBIN
(ACID HEMATIN)
Coleman Universal
Spectrophotometer
13 mm Square Cuvettes
400 mu
Filter PC-4
10
9
8
7
6
5
4
3
2
1
3
6
9
12
15
18
21
gm per 100 ml

Meter Reading	0	1	2	3	4	5	6	7	8	9
0										
10										
20										
30										
40										
50										
60										
70										
80										
90										

HEMOGLOBIN (ALKALINE HEMATIN)

A spectrophotometric adaptation based on the methods and modifications of
Cohen and Smith (1919) and Wu (1922)

PRINCIPLE

Hemoglobin is treated with hydrochloric acid to produce acid hematin which is converted to the alkaline form just before measuring.

DETERMINATION IN WHOLE BLOOD

SAMPLE

Whole blood—either fresh or oxalated samples may be used. This method should be employed only in the absence of abnormal hemoglobin derivatives; if the presence of such derivatives is suspected, Method H-15 should be used.

TEST PROCEDURE

Special Apparatus

Micro pipettes accurately calibrated "to contain" 25 cmm.

Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.

Reagents

1. **Hydrochloric Acid, Volumetric 0.1 N.** Add 10 ml concentrated hydrochloric acid (HCl—Baker & Adamson, A.C.S.) to 1000 ml distilled water and mix; titrate against standard 0.1 N alkali using phenolphthalein as indicator; adjust accordingly and re-titrate—20 ml of 0.1 N acid require 20 ml of 0.1 N alkali.
2. **Sodium Hydroxide, Volumetric 2.5 N.** Prepare by diluting slightly more than the required amount of concentrated carbonate-free sodium hydroxide solution (see Appendix); dilute 20 ml of this approximately 2.5 N solution to 100 ml; titrate against standard 0.5 N acid using phenolphthalein as indicator; adjust accordingly and re-titrate—20 ml of 0.5 N acid require 20 ml of a 1:5 dilution of 2.5 N alkali. Preserve in ceresin-lined bottle.

Technic

1. In a 25 ml volumetric flask (sample) place
   20 ml 0.1 N hydrochloric acid (1).
2. Add 25 cmm fresh or oxalated blood from a micro pipette, thoroughly rinsing out the pipette by drawing in and expelling some of the mixture several times.
3. Stopper the flask and mix the contents by gentle whirling, avoiding excessive foaming.
4. Allow to stand at room temperature for 40 minutes.
5. Add 2.5 ml 2.5 N sodium hydroxide (2).
6. Dilute to volume with 0.1 N hydrochloric acid (1) and mix thoroughly.
7. In a cuvette (reagent blank) place
   9 ml 0.1 N hydrochloric acid (1)
   1 ml 2.5 N sodium hydroxide (2) and mix.
8. Measure the transmittancy of the sample against the reagent blank set at 100% transmission at a wave length of **400** mμ and obtain the concentration of hemoglobin from the calibration table.

CALIBRATION PROCEDURE

Special Apparatus

Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.

Micro pipettes accurately calibrated "to contain" 0.1 ml.

Reagents

1. **Hydrochloric Acid, Volumetric 0.1 N.** Same as for Test Procedure.
2. **Sodium Hydroxide, Volumetric 2.5 N.** Same as for Test Procedure.
3. **Hemoglobin Standards**
   (a) Hemoglobin stock standard. Pool several samples of oxalated blood; centrifuge about 10 ml of the pooled bloods at low speed for 5 minutes; remove and discard about 1 ml of the supernatant plasma and thoroughly mix the remaining plasma and red cells. Determine the total iron content of the concentrated blood and calculate the hemoglobin equivalent as outlined in the methods for total iron (Methods I-27 or I-28). For highest accuracy the average result of several determinations should be used.
   (b) Dilute standard. In a 100 ml volumetric flask place approximately 80 ml 0.1 N hydrochloric acid (1); add 0.1 ml concentrated blood from a micro pipette, thoroughly rinsing out the pipette by drawing in and expelling some of the mixture several times; stopper the flask and mix the contents by gentle whirling, avoiding excessive foaming; allow to stand at room temperature for 40 minutes; add 10 ml 2.5 N sodium hydroxide (2); dilute to volume with 0.1 N hydrochloric acid and mix thoroughly. Prepare just before use and proceed immediately as follows.

Technic

1. Transfer respectively 2.5, 5.0, 7.5 and 10 ml of the dilute standard (3b) to correspondingly marked accurately calibrated 10 ml volumetric flasks (calibration standards).
2. Dilute to volume with 0.1 N hydrochloric acid (1) and mix thoroughly.
3. In a cuvette (reagent blank) place
   9 ml 0.1 N hydrochloric acid (1)
   1 ml 2.5 N sodium hydroxide (2) and mix.
4. Immediately set the reagent blank at 100% transmission at a wave length of **400** mμ and record the transmittancy of the calibration standards.
5. Calculation of the hemoglobin equivalents of the calibration standards—

   Let Hb represent the concentration of hemoglobin in gm per 100 ml in the stock standard (3a)

   Let V represent the volume of the dilute standard (3b) taken for calibration

   Then,

   $$\frac{\text{Hb} \times \text{V}}{10} = \text{Concentration of hemoglobin in the calibration standard}$$

   Example:

   Iron content of stock standard (3a) = 51 mg per 100 ml

   Or, $\frac{51}{3.35}$ = 15.2 gm hemoglobin per 100 ml stock standard (3a)

   Therefore, the dilute standard (3b) has a hemoglobin equivalent of 15.2 gm per 100 ml

   For the calibration standard containing 2.5 ml of the dilute standard, the calculation is —

   $\frac{15.2 \times 2.5}{10}$ or, 3.8 gm hemoglobin per 100 ml blood

   The calculation for the other calibration standards is similar, and in this case, would be 7.6, 11.4 and 15.2 gm per 100 ml.
6. Plot the calculated hemoglobin equivalents of the calibration standards on semi-log graph paper—the points fall on a slight curve which passes through the origin (Calibration Curve H-11). A hemoglobin concentration of 15.6 gm per 100 ml should show a transmittancy reading of approximately 34%.
7. Prepare a calibration table from the T-C values obtained from the plotted curve.

◆

PROCEDURE FOR USE WITH LINEAR-SCALE PHOTOELECTRIC COLORIMETERS (SHEARD-SANFORD TYPE)

TEST PROCEDURE

Exactly as outlined; set the reagent blank at 100 and use a blue filter.

CALIBRATION PROCEDURE

Exactly as outlined—use a blue filter; plot the observed readings on semi-log graph paper—the points fall on a slight curve passing through the origin.

◆

PROCEDURE FOR USE WITH LOGARITHMIC-SCALE PHOTOELECTRIC COLORIMETERS (KLETT-SUMMERSON TYPE)

TEST PROCEDURE

Exactly as outlined; set the reagent blank at 0 and use a green filter.

CALIBRATION PROCEDURE

Exactly as outlined—use a green filter; plot the observed readings on linear graph paper—the points fall on a very slight curve passing through the origin.

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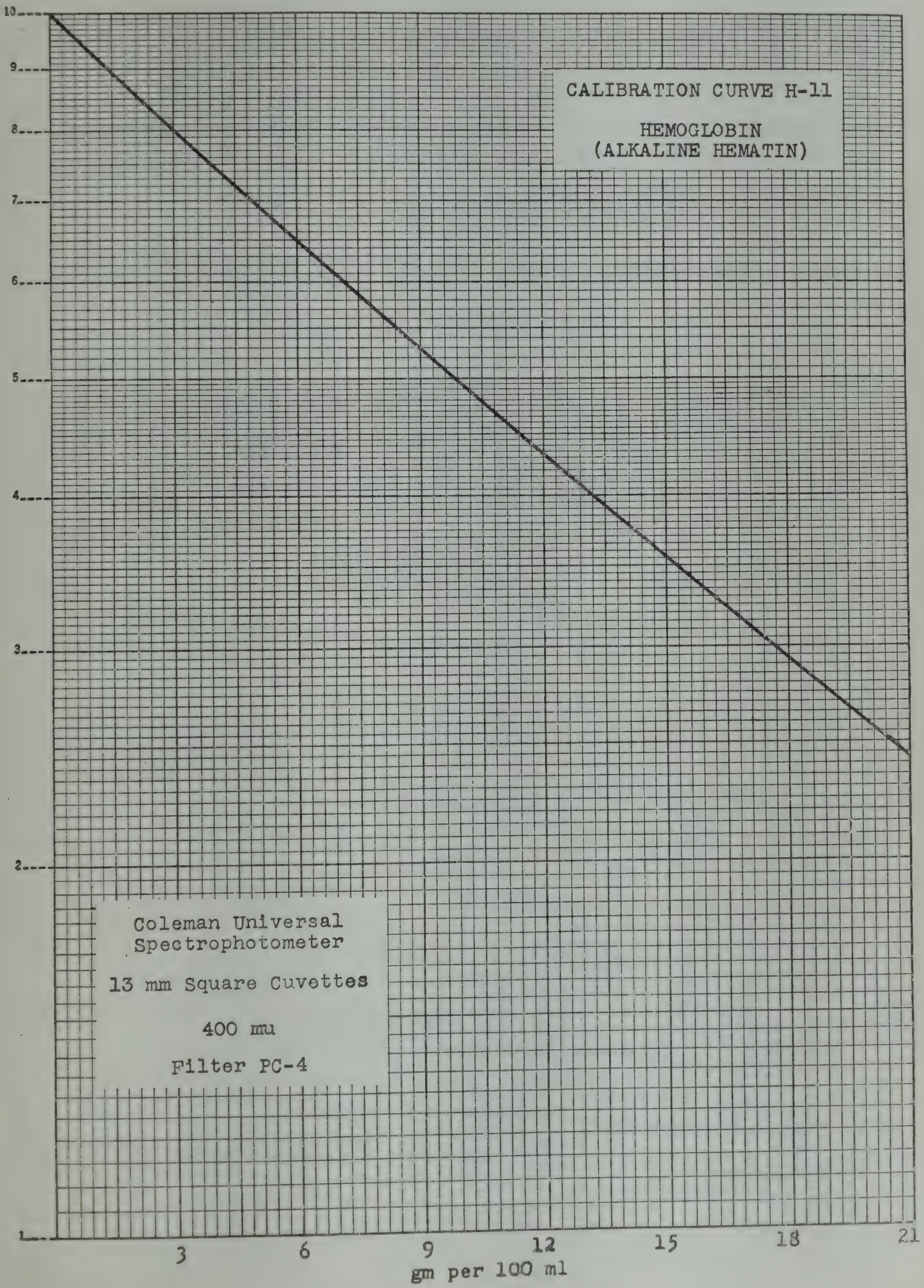
Wu, H., "Studies on Hemoglobin. I. The Advantage of Alkaline Solutions for Colorimetric Methods for the Determination of Hemoglobin," *J. Biochem.*, **2,** 173 (1922).

◆


CALIBRATION CURVE H-11
HEMOGLOBIN
(ALKALINE HEMATIN)
Coleman Universal
Spectrophotometer
13 mm Square Cuvettes
400 mu
Filter PC-4
10
9
8
7
6
5
4
3
2
1
3
6
9
12
15
18
21
gm per 100 ml

Meter Reading	0	1	2	3	4	5	6	7	8	9
0										
10										
20										
30										
40										
50										
60										
70										
80										
90										

HEMOGLOBIN
(Indirect Iron Method)

Hemoglobin may be indirectly estimated as follows by determining the iron content in whole blood—

1. Determine total iron in mg per 100 ml whole blood as outlined in Methods I-27 or I-28.
2. Calculation—

$$\frac{\text{Total iron in mg per 100 ml}}{3.35} = \text{Hemoglobin in gm per 100 ml}$$

Note: The hemoglobin equivalent for the corresponding iron content may be obtained directly from Chart H-12. Locate the determined iron concentration on the vertical axis of the chart, move horizontally across the chart to the diagonal line, then drop to the horizontal axis and obtain the hemoglobin equivalent.

NOTES

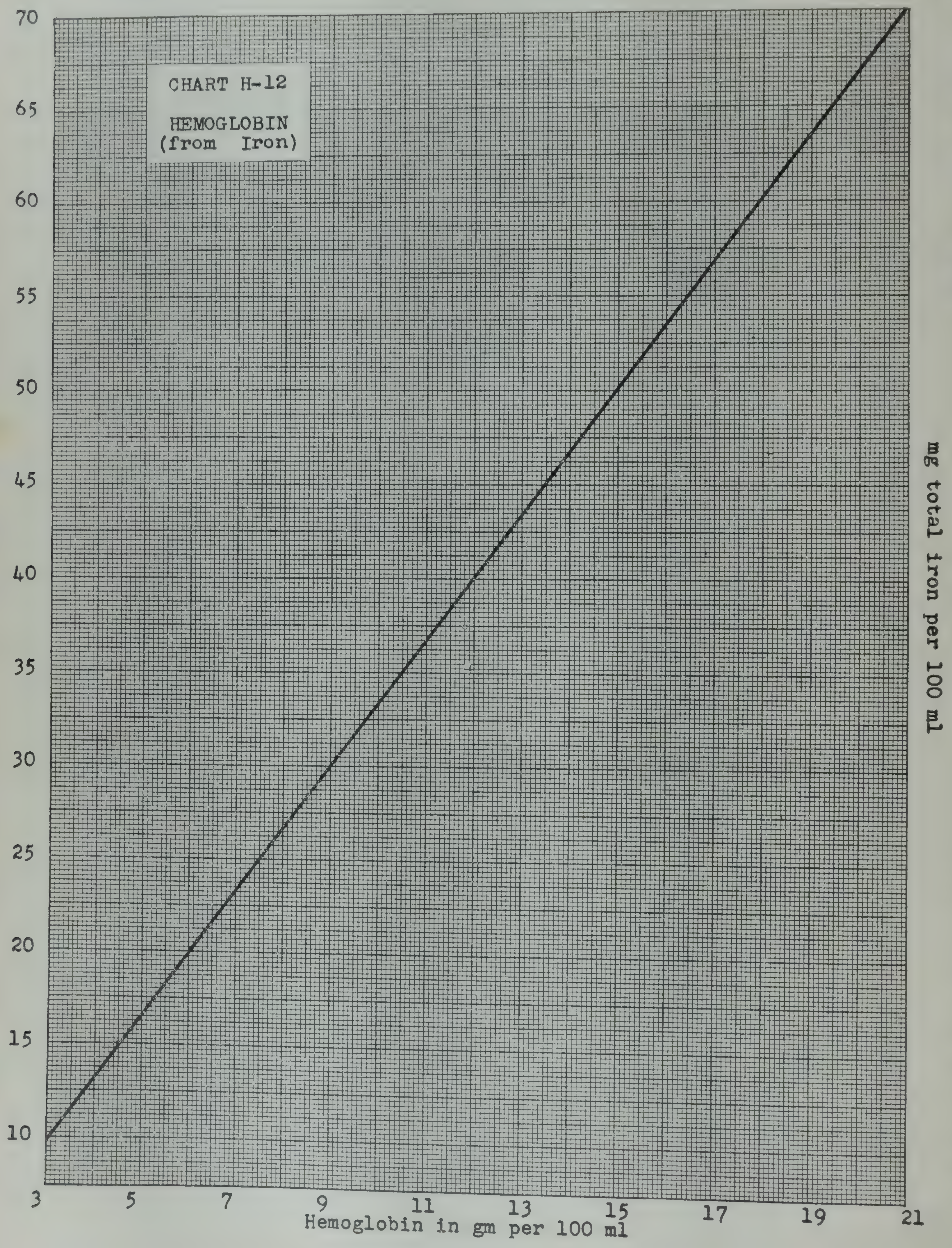


CHART H-12
HEMOGLOBIN
(from Iron)
70
65
60
55
50
45
40
35
30
25
20
15
10
3
5
7
9
11
13
15
17
19
21
Hemoglobin in gm per 100 ml
mg total iron per 100 ml

HEMOGLOBIN
(Indirect Oxygen Capacity Method)

Hemoglobin may be indirectly estimated as follows by determining the oxygen capacity of whole blood—

1. Determine the oxygen capacity in volumes per cent whole blood as outlined in Method O–10.
2. Calculation—

$$\frac{\text{Oxygen capacity in volumes per cent}}{1.36} = \text{Hemoglobin in gm per 100 ml}$$

Note: The hemoglobin equivalent for the corresponding oxygen capacity may be obtained directly from Chart H-13. Locate the determined oxygen capacity on the vertical axis of the chart, move horizontally across the chart to the diagonal line, then drop to the horizontal axis and obtain the hemoglobin equivalent.

NOTES

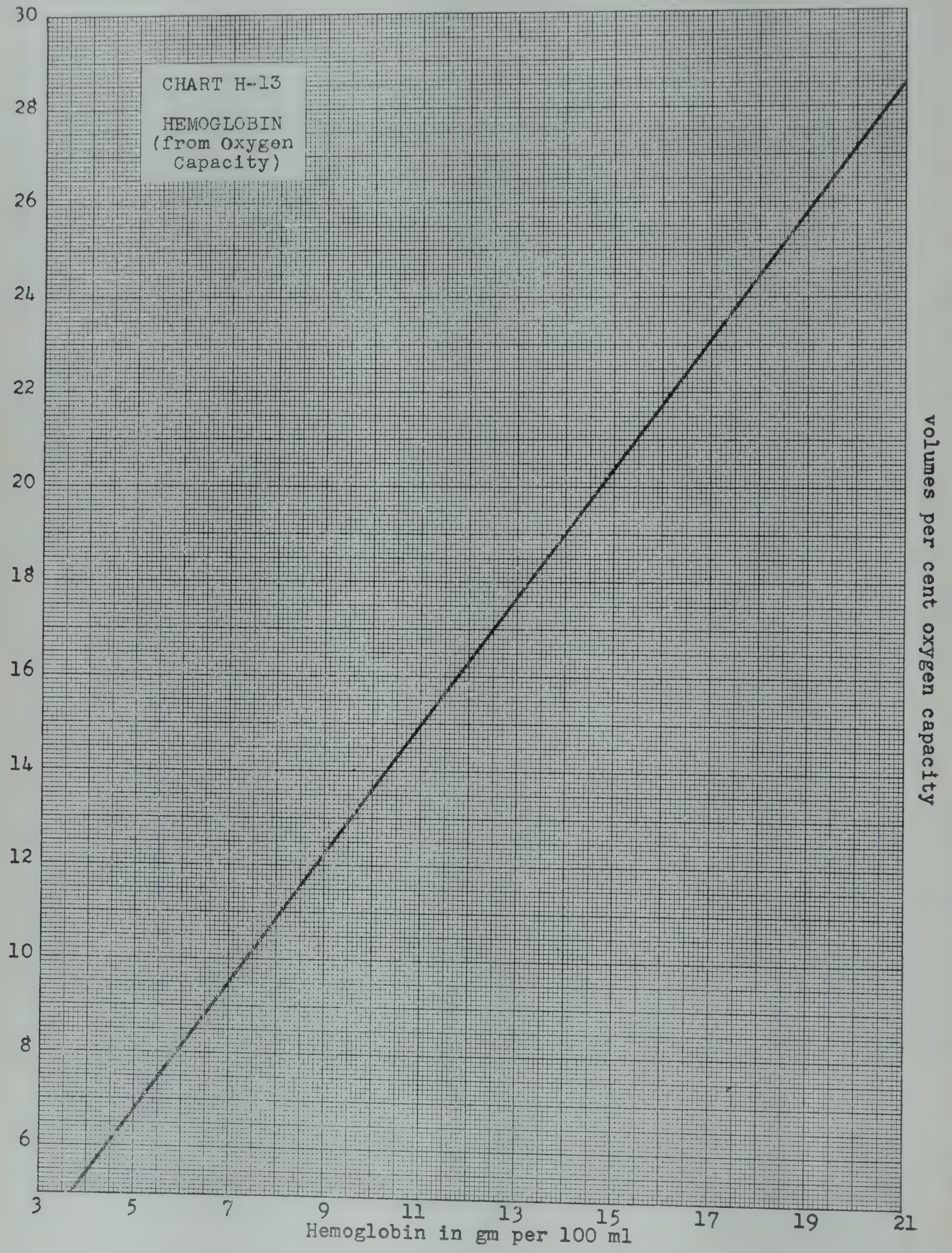


CHART H-13
HEMOGLOBIN
(from Oxygen
Capacity)
30
28
26
24
22
20
18
16
14
12
10
8
6
3
5
7
9
11
13
15
17
19
21
Hemoglobin in gm per 100 ml
volumes per cent oxygen capacity

HEMOGLOBIN (OXYHEMOGLOBIN)

A spectrophotometric adaptation based on the method of Sheard and Sanford (1929)

PRINCIPLE

Hemoglobin is determined by the reddish color of oxyhemoglobin produced upon treatment of the blood with sodium carbonate.

DETERMINATION IN WHOLE BLOOD

SAMPLE

Whole blood—either fresh or oxalated samples may be used. This method should be employed only in the absence of abnormal hemoglobin derivatives; if the presence of such derivatives is suspected, Method H-15 should be used.

TEST PROCEDURE

Special Apparatus

Micro pipettes accurately calibrated "to contain" 20 cmm.

Reagent

1. **Sodium Carbonate, 0.1% Aqueous.** Dissolve 1 gm anhydrous sodium carbonate (Na_2CO_3—Baker & Adamson, A.C.S.) in distilled water and dilute to 1000 ml. Preserve in ceresin-lined bottle.

Technic

1. In a cuvette (sample) place
   5 ml 0.1% sodium carbonate (1).
2. Add 20 cmm fresh or oxalated blood from a micro pipette, thoroughly rinsing out the pipette by drawing in and expelling some of the mixture several times.
3. Mix thoroughly by shaking for 10 seconds.
4. Measure the transmittancy of the sample against 0.1% sodium carbonate (1) set at 100% transmission at a wave length of **545 mμ** and obtain the concentration of hemoglobin from the calibration table.

CALIBRATION PROCEDURE

Special Apparatus

Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.

Micro pipettes accurately calibrated "to contain" 0.1 ml.

Reagents

1. **Sodium Carbonate, 0.1% Aqueous.** Same as for Test Procedure.
2. **Hemoglobin Standards**
   (a) Hemoglobin stock standard. Pool several samples of oxalated blood; centrifuge about 10 ml of the pooled bloods at low speed for 5 minutes; remove and discard about 1 ml of the supernatant plasma and thoroughly mix the remaining plasma and red cells. Determine the total iron content of the concentrated blood and calculate the hemoglobin equivalent as outlined in the methods for total iron (Methods I-27 or I-28). For highest accuracy the average result of several determinations should be used.
   (b) Dilute standard. In a 25 ml volumetric flask place 25 ml 0.1% sodium carbonate (1); add 0.1 ml concentrated blood from a micro pipette, thoroughly rinsing out the pipette by drawing in and expelling some of the mixture several times; mix thoroughly. Prepare just before use and proceed immediately as follows.

Technic

1. Transfer respectively 2.5, 5.0, 7.5 and 10 ml of the dilute standard (2b) to correspondingly marked accurately calibrated 10 ml volumetric flasks (calibration standards).
2. Dilute to volume with 0.1% sodium carbonate (1) and mix thoroughly.
3. Immediately set 0.1% sodium carbonate (1) at 100% transmission at a wave length of **545 mμ** and record the transmittancy of the calibration standards.
4. Calculation of the hemoglobin equivalents of the calibration standards—
   Let Hb represent the concentration of hemoglobin in gm per 100 ml in the stock standard (2a)
   Let V represent the volume of the dilute standard (2b) taken for calibration
   Then,
   $\frac{Hb \times V}{10}$ = Concentration of hemoglobin in the calibration standard
   Example:
   Iron content of stock standard (2a) = 51 mg per 100 ml
   Or, $\frac{51}{3.35}$ = 15.2 gm hemoglobin per 100 ml stock standard (2a)
   Therefore, the dilute standard (2b) has a hemoglobin equivalent of 15.2 gm per 100 ml

For the calibration standard containing 2.5 ml of the dilute standard, the calculation is—$\frac{15.2 \times 2.5}{10}$ or, 3.8 gm hemoglobin per 100 ml blood

The calculation for the other calibration standards is similar and, in this case, would be 7.6, 11.4 and 15.2 gm per 100 ml.

5. Plot the calculated hemoglobin equivalents of the calibration standards on semi-log graph paper—the points fall on a slight curve which passes through the origin (Calibration Curve H-14). A hemoglobin concentration of 15.6 gm per 100 ml should show a transmittancy reading of approximately 34%.
6. Prepare a calibration table from the T-C values obtained from the plotted curve.

PROCEDURE FOR USE WITH LINEAR-SCALE PHOTOELECTRIC COLORIMETERS (SHEARD-SANFORD TYPE)

TEST PROCEDURE

Exactly as outlined; set 0.1% sodium carbonate at 100 and use a green filter.

CALIBRATION PROCEDURE

Exactly as outlined—use a green filter; plot the observed readings on semi-log graph paper—the points fall on a very slight curve passing through the origin.

PROCEDURE FOR USE WITH LOGARITHMIC-SCALE PHOTOELECTRIC COLORIMETERS (KLETT-SUMMERSON TYPE)

TEST PROCEDURE

Exactly as outlined; set 0.1% sodium carbonate at 0 and use a green filter.

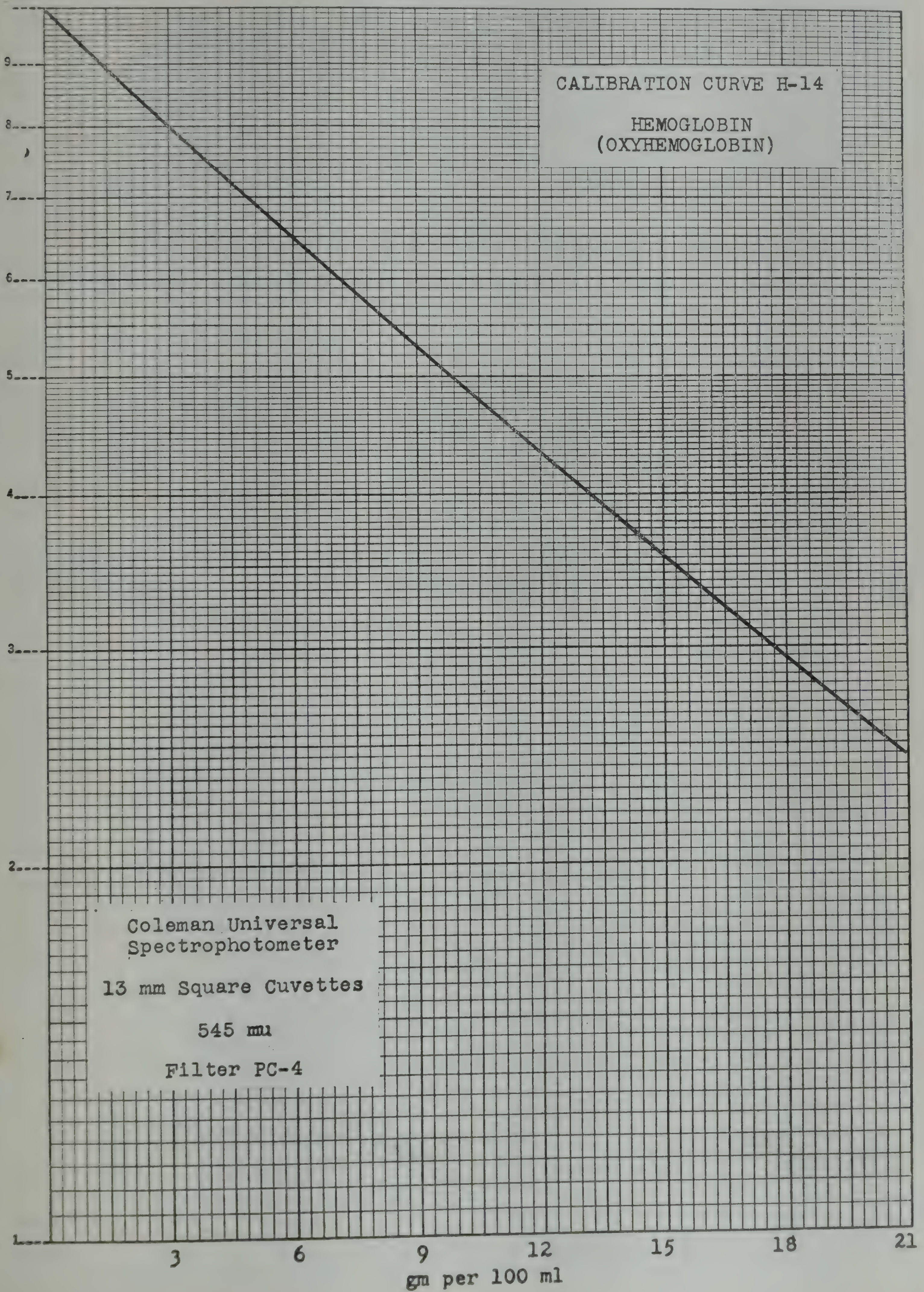
CALIBRATION PROCEDURE

Exactly as outlined—use a green filter; plot the observed readings on linear graph paper—the points fall on a very slight curve passing through the origin.

BIBLIOGRAPHY

Sheard, C., and Sanford, A. H., "A Photoelectric Hemoglobinometer; Clinical Applications of the Principles of Photoelectric Photometry to the Measurement of Hemoglobin," *J. Lab. Clin. Med.*, 14, 558 (1929).

NOTES


CALIBRATION CURVE H-14
HEMOGLOBIN
(OXYHEMOGLOBIN)
Coleman Universal Spectrophotometer
13 mm Square Cuvettes
545 mμ
Filter PC-4
9
8
7
6
5
4
3
2
1
3
6
9
12
15
18
21
gm per 100 ml

Meter Reading	0	1	2	3	4	5	6	7	8	9
0										
10										
20										
30										
40										
50										
60										
70										
80										
90										

HEMOGLOBIN, TOTAL

A spectrophotometric adaptation based on the methods and modifications of Stadie (1920), Wu (1922) and Evelyn and Malloy (1938)

PRINCIPLE

Blood is hemolyzed in a phosphate buffer and treated with potassium ferricyanide to convert the hemoglobin to methemoglobin. Upon further treatment with potassium cyanide the methemoglobin forms cyanhemoglobin. Clarification and the elimination of interference by non-hemoglobin substances in the plasma and red cell stroma is accomplished by the addition of ammonia.

DETERMINATION IN WHOLE BLOOD

SAMPLE

Whole blood—either fresh or oxalated samples may be used. This method should be employed if the presence of abnormal hemoglobin derivatives is suspected.

TEST PROCEDURE

Special Apparatus

Micro pipettes accurately calibrated "to contain" 20 cmm.

Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.

Reagents

1. **Phosphate Buffer Solution, M/60, pH 6.0 (Evelyn-Malloy).** Dilute 25 ml M/15 phosphate buffer solution (a) to 100 ml with distilled water. Prepare fresh daily and keep in resistant-glass bottle.
    - (a) Phosphate buffer solution, M/15 (Soerensen); mix 12 ml M/15 disodium phosphate (i) and 88 ml M/15 monopotassium phosphate (ii); preserve in resistant-glass bottle.
        - (i) Disodium phosphate, M/15 (Soerensen); dry anhydrous disodium phosphate (Na_2HPO_4—Merck, Special for pH) at 100° C. for one hour and cool in desiccator to constant weight; transfer 9.47 gm to a 1000 ml volumetric flask; add approximately 900 ml freshly boiled and cooled distilled water and shake until dissolved; dilute to volume; add 5 ml chloroform and preserve in resistant-glass bottle.
        - (ii) Monopotassium phosphate, M/15 (Soerensen); dry crystalline monopotassium phosphate (KH_2PO_4—Merck, Reagent, Soerensen) at 100° C. for one hour and cool in desiccator to constant weight; transfer 9.08 gm to a 1000 ml volumetric flask; add approximately 900 ml freshly boiled and cooled distilled water and shake until dissolved; dilute to volume; add 5 ml chloroform and preserve in resistant-glass bottle.
2. **Potassium Ferricyanide, 20% Aqueous.** Dissolve 5 gm potassium ferricyanide ($K_3Fe(CN)_6$—Baker & Adamson, A.C.S.) in distilled water and dilute to 25 ml. Preserve in a brown glass bottle equipped with a dropper delivering 25 drops per ml. Prepare fresh frequently.
3. **Potassium Cyanide, 5% Aqueous.** Dissolve 1 gm granular potassium cyanide (KCN—Baker & Adamson, Reagent) in distilled water and dilute to 20 ml. Preserve in a brown glass bottle equipped with a dropper delivering 25 drops per ml. Prepare fresh frequently.
4. **Ammonium Hydroxide, Concentrated** (NH_4OH—Baker & Adamson, A.C.S.). Transfer a small amount to a brown glass bottle equipped with a dropper delivering 25 drops per ml.

Technic

1. In a cuvette (sample) place
   6 ml M/60 phosphate buffer solution (1).
2. Add 20 cmm fresh or oxalated blood from a micro pipette, thoroughly rinsing out the pipette by drawing in and expelling some of the mixture several times; allow to stand for 2 minutes.
3. In another cuvette (reagent blank) place
   6 ml M/60 phosphate buffer solution (1).
4. To each cuvette add 1 drop 20% potassium ferricyanide (2), mix and allow to stand for 10 minutes.
5. Add 1 drop 5% potassium cyanide (3), mix and allow to stand for 2 minutes.
6. Add 1 drop concentrated ammonium hydroxide (4) and mix.
7. Within 10 minutes, measure the transmittancy of the sample against the reagent blank set at 100% transmission at a wave length of 540 mμ and obtain the concentration of total hemoglobin from the calibration table.

CALIBRATION PROCEDURE

Special Apparatus

Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.

Micro pipettes accurately calibrated "to contain" 0.1 ml.

Reagents

1. **Phosphate Buffer Solution, M/60, pH 6.0 (Evelyn-Malloy).** Same as for Test Procedure.
2. **Potassium Ferricyanide, 20% Aqueous.** Same as for Test Procedure.
3. **Potassium Cyanide, 5% Aqueous.** Same as for Test Procedure.
4. **Ammonium Hydroxide, Concentrated.** Same as for Test Procedure.
5. **Hemoglobin Standards**
    (a) Hemoglobin stock standard. Pool several samples of oxalated blood; centrifuge about 10 ml of the pooled bloods at low speed for 5 minutes; remove and discard about 1 ml of the supernatant plasma and thoroughly mix the remaining plasma and red cells. Determine the total iron content of the concentrated blood and calculate the hemoglobin equivalent as outlined in the methods for total iron (Methods I-27 or I-28). For highest accuracy the average result of several determinations should be used.
    (b) Dilute standard. In a 50 ml Erlenmeyer flask place exactly 30 ml M/60 phosphate buffer solution (1); add 0.1 ml concentrated blood from a micro pipette, thoroughly rinsing out the pipette by drawing in and expelling some of the mixture several times; mix and allow to stand for 2 minutes; add 5 drops 20% potassium ferricyanide (2), mix and allow to stand for 10 minutes; add 5 drops 5% potassium cyanide (3), mix and allow to stand for 2 minutes; add 5 drops concentrated ammonium hydroxide (4) and mix. Prepare just before use and proceed immediately as follows.

Technic

1. In a 25 ml mixing cylinder (reagent blank) place exactly
    24 ml M/60 phosphate buffer solution (1)
    4 drops 20% potassium ferricyanide (2) and mix
    4 drops 5% potassium cyanide (3) and mix
    4 drops concentrated ammonium hydroxide (4) and mix.
2. Transfer respectively 2.5, 5.0, 7.5 and 10 ml of the dilute standard (5b) to correspondingly marked accurately calibrated 10 ml volumetric flasks (calibration standards).
3. Dilute to volume with the reagent blank solution prepared in Step 1.
4. Immediately set the reagent blank at 100% transmission at a wave length of 540 mμ and record the transmittancy of the calibration standards—readings should be completed within 10 minutes after preparation of the dilute standard (5b).
5. Calculation of the hemoglobin equivalents of the calibration standards—
    Let Hb represent the concentration of hemoglobin in gm per 100 ml in the stock standard (5a)
    Let V represent the volume of the dilute standard (5b) taken for calibration
    Then,
    $$\frac{Hb \times V}{10} = \text{Concentration of hemoglobin in the calibration standard}$$
    Example:
    Iron content of stock standard (5a) = 51 mg per 100 ml
    $$\text{Or, } \frac{51}{3.35} = 15.2 \text{ gm hemoglobin per 100 ml stock standard (5a)}$$
    Therefore, the dilute standard (5b) has a hemoglobin equivalent of 15.2 gm per 100 ml
    For the calibration standard containing 2.5 ml of the dilute standard, the calculation is —
    $$\frac{15.2 \times 2.5}{10} \text{ or, 3.8 gm hemoglobin per 100 ml blood}$$
    The calculation for the other calibration standards is similar and in this case, would be 7.6, 11.4 and 15.2 gm per 100 ml.
6. Plot the calculated hemoglobin equivalents of the calibration standards on semi-log graph paper—the points fall on a straight line which passes through the origin (Calibration Curve H-15). A hemoglobin concentration of 15.6 gm per 100 ml should show a transmittancy reading of approximately 43%.
7. Prepare a calibration table from the T-C values obtained from the plotted curve.

PROCEDURE FOR USE WITH LINEAR-SCALE PHOTOELECTRIC COLORIMETERS (SHEARD-SANFORD TYPE)

TEST PROCEDURE

Exactly as outlined; set the reagent blank at 100 and use a green filter.

CALIBRATION PROCEDURE

Exactly as outlined—use a green filter; plot the observed readings on semi-log graph paper—the points fall on a straight line passing through the origin.

PROCEDURE FOR USE WITH LOGARITHMIC-SCALE PHOTOELECTRIC COLORIMETERS (KLETT-SUMMERSON TYPE)

TEST PROCEDURE

Exactly as outlined; set the reagent blank at 0 and use a green filter.

CALIBRATION PROCEDURE

Exactly as outlined—use a green filter; plot the observed readings on linear graph paper—the points fall on a straight line passing through the origin.

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NOTES

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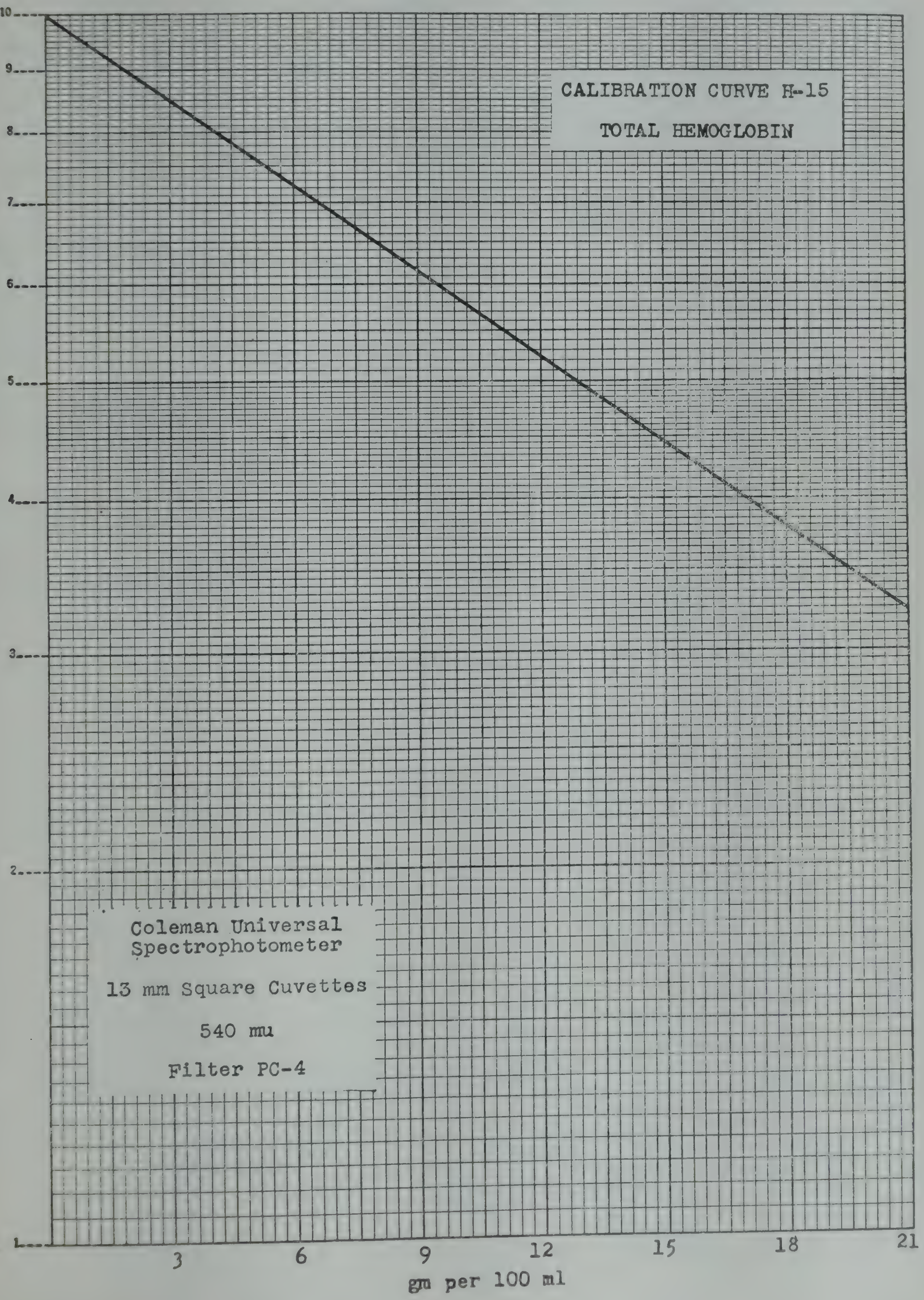

CALIBRATION CURVE H-15
TOTAL HEMOGLOBIN
Coleman Universal Spectrophotometer
13 mm Square Cuvettes
540 mu
Filter PC-4
10
9
8
7
6
5
4
3
2
1
3
6
9
12
15
18
21
gm per 100 ml

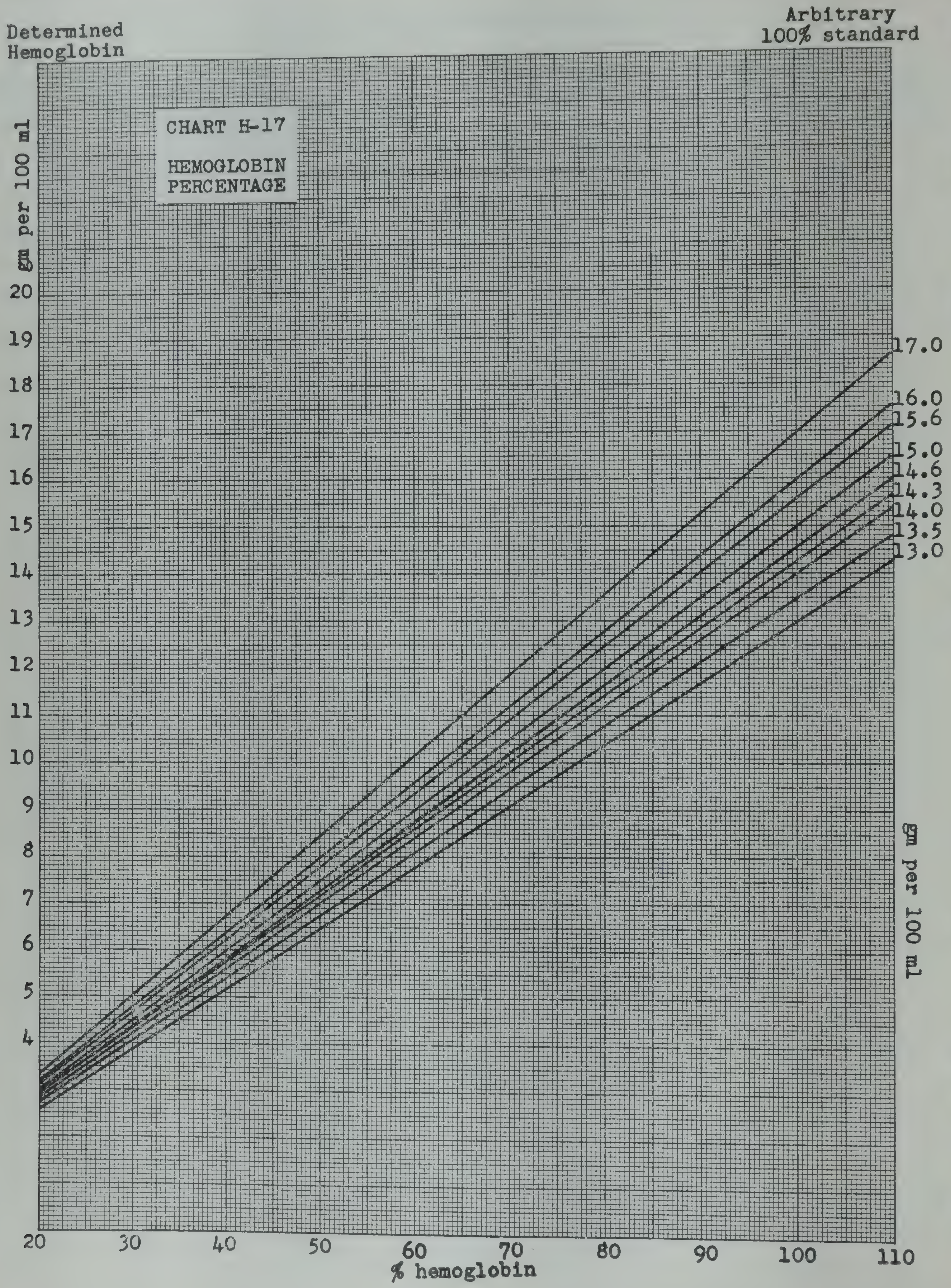
Meter Reading	0	1	2	3	4	5	6	7	8	9
0										
10										
20										
30										
40										
50										
60										
70										
80										
90										

HEMOGLOBIN PERCENTAGE

The percentage of the determined hemoglobin as compared with an arbitrary normal standard may be obtained directly from Chart H-17.

Locate the determined hemoglobin concentration on the vertical axis of the chart, move horizontally across the chart to the diagonal line representing the selected standard, then drop to the horizontal axis and obtain the hemoglobin percentage.

NOTES


Determined Hemoglobin
gm per 100 ml
Arbitrary 100% standard
CHART H-17
HEMOGLOBIN PERCENTAGE
20
19
18
17
16
15
14
13
12
11
10
9
8
7
6
5
4
17.0
16.0
15.6
15.0
14.6
14.3
14.0
13.5
13.0
gm per 100 ml
20
30
40
50
60
70
80
90
100
110
% hemoglobin

HIPPURIC ACID

A spectrophotometric adaptation based on the methods and modifications of Quick (1933), Denigès (1940) and Deysson and Alliot(1947)

PRINCIPLE

When hippuric acid is heated with sodium hypobromite, a red precipitate of bromobenzamide forms (Denigès reaction) which is then extracted with amyl alcohol and the resultant reddish-orange color used for spectrophotometric measurement.

DETERMINATION IN URINE

SAMPLE

Aliquot of a 4-hour specimen (oral technic) or a 1-hour specimen (intravenous technic). No drugs must be administered for 48 hours prior to the test. Benzoic acid, salicyluric acid and salicylamide do not interfere. The amount of diluted urine taken for analysis must contain less than 15 mg of urea.

TEST PROCEDURE

Special Apparatus

Graduated test tubes—25 ml in 0.5 ml.
Capillary pipettes with rubber bulbs.
Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.

Reagents

1. **Sodium Hypobromite Reagent.** Just prior to use, mix equal volumes of 10 N sodium hydroxide (a) and bromine-bromide solution (b). Prepare only the amount required and discard any excess.
   - (a) Sodium hydroxide, volumetric 10 N; prepare by diluting slightly more than the required amount of concentrated carbonate-free sodium hydroxide solution (see Appendix); dilute 1 ml of this approximately 10 N solution to 100 ml; titrate against standard 0.1 N acid using phenolphthalein as indicator; adjust accordingly and retitrate—20 ml of 0.1 N acid require 20 ml of a 1:100 dilution of 10 N alkali. Preserve in ceresin-lined bottle.
   - (b) Bromine-bromide solution (Deysson-Alliot); in a 100 ml mixing cylinder place 85 ml distilled water; add 50 gm potassium bromide (KBr—Baker & Adamson, A.C.S.) and shake until dissolved; add *exactly* 8.5 ml bromine (Br—Baker & Adamson, A.C.S.) from a burette and mix. Preserve in a glass-stoppered bottle. Note: The bromine must be measured with the greatest possible accuracy.
2. **iso-Amyl Alcohol** ($C_6H_{11}OH$—Baker & Adamson, A.C.S.).

Technic

Note: For a hippuric acid content of less than 1 mg in the sample taken for analysis, dilute a larger volume of urine to 10 ml in Step 3; for concentrations in excess of 5 mg in the sample taken for analysis, dilute less urine to 10 ml in Step 3; in either case, correct the results accordingly.

The accuracy and reproducibility of the procedure is dependent upon precise measurement of the hypobromite reagent.

1. Note the total volume of urine voided and, by means of a hydrometer, measure the specific gravity of the combined urine specimens and record.
2. Filter a small portion of thoroughly mixed urine.
3. Dilute 2 ml filtered urine to 10 ml with distilled water.
4. In a 25 ml graduated test tube (sample) place an amount of diluted urine determined as follows:

Specific Gravity of Original Specimen	Amount of Diluted Urine to Be Taken for Analysis
1.001–1.005	5 ml
1.006–1.010	4 ml
1.011–1.015	3 ml
1.016–1.020	2 ml
1.021–1.025	1 ml
1.026–1.030	0.5 ml

5. Dilute to 5 ml with distilled water.
6. Add 12 ml sodium hypobromite reagent (1) and mix.
7. In another 25 ml graduated test tube (sample blank) place the same volume of diluted urine as taken in Step 4; add 12 ml distilled water and mix.
8. Place the tubes in actively boiling water for 5 minutes.
9. Cool in running tap water for 3 minutes.
10. To each tube add 6 ml iso-amyl alcohol (2), stopper the tubes and shake vigorously for 30 seconds.
11. Allow to stand until the alcohol layers separate.
12. Withdraw as much as possible of each alcohol layer by means of capillary pipettes with rubber bulbs and transfer through small Whatman #40 papers into correspondingly marked dry cuvettes—cloudy alcohol extracts may be cleared by centrifuging briefly.
13. Measure the transmittancy of the sample against the sample blank set at 100% transmission at a wave length of 400 mμ, obtain the value from the calibration table and calculate the concentration of hippuric acid as follows.

Calculation

$$\text{Value from calibration table} \times \frac{500}{\text{Amount of diluted urine taken for analysis (from Step 4)}} = \text{Hippuric acid in mg per 100 ml}$$

$$\frac{\text{Total volume of voided specimen}}{100} \times \text{Hippuric acid in mg per 100 ml} = \text{Mg hippuric acid excreted during test period}$$

CALIBRATION PROCEDURE

Special Apparatus

Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.
Graduated test tubes—25 ml in 0.5 ml.
Capillary pipettes with rubber bulbs.

Reagents

1. **Sodium Hypobromite Reagent.** Same as for Test Procedure.
2. **iso-Amyl Alcohol.** Same as for Test Procedure.
3. **Hippuric Acid Standards**
   (a) Hippuric acid stock standard (1 ml contains 1 mg hippuric acid). In a 100 ml volumetric flask place 100 mg hippuric acid ($C_6H_5CONHCH_2COOH$—Pfanstiehl, C.P.); add approximately 80 ml distilled water and 1 ml 1 N sodium hydroxide; shake until dissolved and dilute to volume with distilled water.
   (b) Calibration standards. In a series of six 25 ml graduated test tubes place respectively 0, 1, 2, 3, 4 and 5 ml of the stock standard (3a); add 5, 4, 3, 2, 1 and 0 ml distilled water respectively, bringing the volume in each tube to 5 ml. These standards contain concentrations of hippuric acid equivalent to 0, 1, 2, 3, 4 and 5 mg per 5 ml. Prepare just before use and proceed immediately as follows.

Technic

1. To each calibration standard (3b) add 12 ml sodium hypobromite reagent (1) and mix.
2. Proceed exactly as outlined in the Test Procedure, Steps 8–12 inclusive.
3. Set the zero concentration standard at 100% transmission at a wave length of 400 mμ and record the transmittancy of the 1, 2, 3, 4 and 5 mg standards. The transmittancy of the 5 mg standard should be approximately 25.5%.
4. Plot the observed values on semi-log graph paper—the points fall on a straight line which passes through the concentration axis to the right of the origin (Calibration Curve H-20).
5. Prepare a calibration table from the T-C values obtained from the plotted curve.

PROCEDURE FOR USE WITH LINEAR-SCALE PHOTOELECTRIC COLORIMETERS (SHEARD-SANFORD TYPE)

TEST PROCEDURE

Exactly as outlined; set the sample blank at 100 and use a green filter.

CALIBRATION PROCEDURE

Exactly as outlined—use a green filter; plot the observed readings on semi-log graph paper—the points fall on a straight line passing through the concentration axis to the right of the origin.

PROCEDURE FOR USE WITH LOGARITHMIC-SCALE PHOTOELECTRIC COLORIMETERS (KLETT-SUMMERSON TYPE)

TEST PROCEDURE

Exactly as outlined; set the sample blank at 0 and use a green filter.

CALIBRATION PROCEDURE

Exactly as outlined—use a green filter; plot the observed readings on linear graph paper—the points fall on a straight line passing through the concentration axis to the right of the origin.

Note: For instruments with cells, cuvettes or tubes requiring a final volume of more than 6 ml, increase the amount of amyl alcohol to the volume required for measurement in both the test and calibration procedures.

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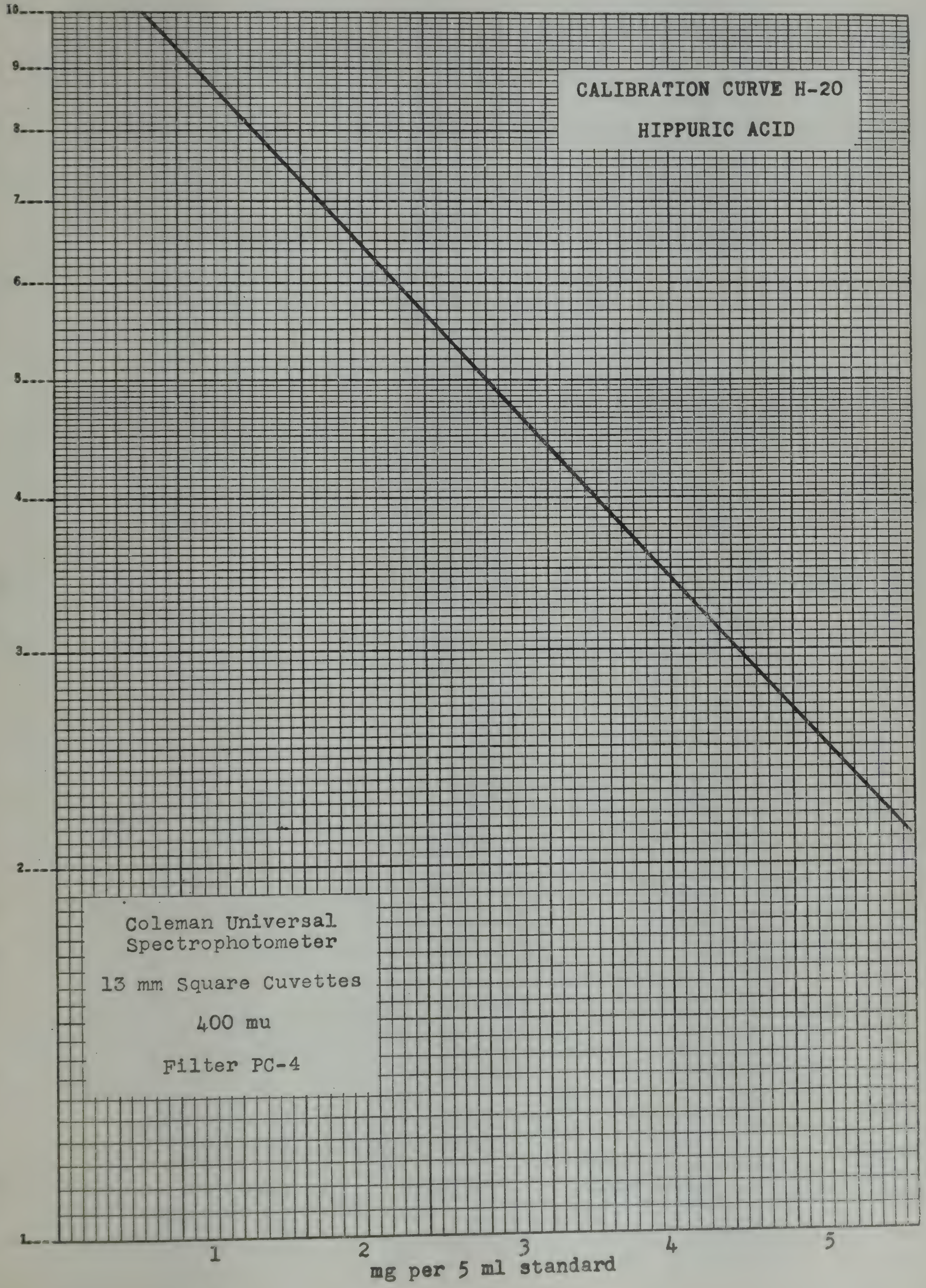
CALIBRATION CURVE H-20
HIPPURIC ACID
Coleman Universal Spectrophotometer
13 mm Square Cuvettes
400 mu
Filter PC-4
10
9
8
7
6
5
4
3
2
1
1
2
3
4
5
mg per 5 ml standard

Meter Reading	0	1	2	3	4	5	6	7	8	9
0										
10										
20										
30										
40										
50										
60										
70										
80										
90										

INDICAN

A spectrophotometric adaptation based on the methods and modifications of Sharlit (1933; 1934; 1935); Townsend (1938) and Meiklejohn and Cohen (1942)

PRINCIPLE

Indican (indoxyl potassium sulfate) forms a reddish-purple condensation product with thymol (Jolles reaction). Persulfate is used to prevent any pigment formation by the closely related skatoxyl derivatives. Ethyl trichloracetate, formed during the condensation process, settles out and at the same time extracts the pigment from the aqueous phase. The trichloracetate layer is taken up in acetic acid and used for spectrophotometric measurement.

DETERMINATION IN PLASMA OR SERUM

SAMPLE

Plasma or serum from blood withdrawn during post-absorptive state. Anticoagulant for plasma—lithium oxalate; potassium oxalate should not be used.

TEST PROCEDURE

Special Apparatus

Ostwald-Folin pipettes accurately calibrated "to contain" 4 ml.

Graduated centrifuge tubes, Pyrex; 15 ml in 0.1 ml; the 10 ml graduation must be accurately calibrated.

Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.

Reagents

1. **Trichloracetic Acid, 25% Aqueous.** Dilute 25 ml concentrated trichloracetic acid (a) to 100 ml with distilled water. Preserve in glass-stoppered bottle. Prepare fresh monthly.
   (a) Trichloracetic acid, concentrated; dissolve 100 gm crystalline trichloracetic acid (CCl_3COOH—J. T. Baker, CP Analyzed) in distilled water and dilute to 100 ml. Preserve in glass-stoppered bottle. Stability indefinite.
2. **Potassium Persulfate, 1% Aqueous.** Dissolve 1 gm crystalline potassium persulfate ($K_2S_2O_8$—Baker & Adamson, Reagent) in distilled water and dilute to 100 ml. Preserve in refrigerator. Prepare fresh weekly.
3. **Thymol, 1% Alcoholic.** Dissolve 1 gm thymol ($C_{10}H_{14}O$—Merck, Reagent) in 100 ml 95% ethyl alcohol (Reagent #4). Preserve in brown glass bottle.
4. **Ethyl Alcohol, 95%** (C_2H_5OH—U. S. Industrial Chemicals, U.S.P.).
5. **Trichloroacetic-Hydrochloric Acid Reagent (Sharlit).** Add 12.5 gm crystalline trichloracetic acid (CCl_3COOH—J. T. Baker, CP Analyzed) to 100 ml concentrated hydrochloric acid (HCl—Baker & Adamson, A.C.S.); crush the crystals with a footed glass rod and stir until dissolved. Preserve in glass-stoppered bottle.
6. **Acetic Acid, Glacial** (CH_3COOH—Baker & Adamson, A.C.S.).

Technic

1. In a 25 ml centrifuge tube place
   6 ml distilled water
   4 ml sample from an Ostwald-Folin pipette, thoroughly rinsing out the pipette by drawing in and expelling some of the mixture several times.
2. From a burette add 10 ml 25% trichloracetic acid (1) in the following manner—three successive additions of 5 drops each, stirring thoroughly between each addition with a thin glass rod for 15 seconds; add the remainder in two equal portions, again thoroughly stirring after each addition.
3. Allow to stand for 5 minutes.
4. Centrifuge at 1800 rpm for 10 minutes.
5. Decant the centrifugate through a dry 9 cm Whatman #40 paper into a dry test tube.
6. In a 15 ml Pyrex graduated centrifuge tube (sample) place
   5 ml filtrate
   0.2 ml 1% potassium persulfate (2)
   0.5 ml 1% thymol (3) and mix.
7. In another 15 ml Pyrex graduated centrifuge tube (sample blank) place
   5 ml filtrate
   0.2 ml 1% potassium persulfate (2).
   0.5 ml 95% ethyl alcohol (4) and mix.
8. To each tube add 5 ml trichloracetic-hydrochloric acid reagent (5), stopper with clean corks and mix thoroughly by inversion.
9. Allow to stand at room temperature for 15 minutes.
10. Place the tubes in vigorously boiling water for 12 minutes.
11. Remove tubes from the water bath and allow to stand at room temperature for 25 minutes.
12. Centrifuge at 1800 rpm for 10 minutes.
13. By means of a capillary pipette, carefully draw off as much of the upper aqueous phase as possible and discard—not more than 0.3 ml of the aqueous phase should remain.
14. Dilute to exactly 10 ml with glacial acetic acid (6) and stir with thin glass rods.

15. Measure the transmittancy of the sample against the sample blank set at 100% transmission at a wave length of **540** mμ and obtain the concentration of indican from the calibration table.

CALIBRATION PROCEDURE

Special Apparatus

Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.

Graduated centrifuge tubes, Pyrex; 15 ml in 0.1 ml; the 10 ml graduation must be accurately calibrated.

Reagents

1. **Trichloracetic Acid, 25% Aqueous.** Same as for Test Procedure.
2. **Potassium Persulfate, 1% Aqueous.** Same as for Test Procedure.
3. **Thymol, 1% Alcoholic.** Same as for Test Procedure.
5. **Trichloracetic-Hydrochloric Acid Reagent (Sharlit).** Same as for Test Procedure.
6. **Acetic Acid, Glacial.** Same as for Test Procedure.
7. **Indican Standards**
    (a) Indican stock standard (1 ml contains 1 mg indican). In a 100 ml volumetric flask place 100 mg potassium indoxyl sulfate ($C_8H_6N \cdot SO_4K$—Reagent Chemicals, Special); add approximately 80 ml distilled water; shake until dissolved and dilute to volume. Preserve in a brown glass bottle in refrigerator.
    (b) Dilute standard (1 ml contains 0.1 mg indican). In a 100 ml volumetric flask place 10 ml stock standard (7a) and dilute to volume with distilled water. Prepare just before use.
    (c) Calibration standards. In a series of seven accurately calibrated 10 ml volumetric flasks place respectively 0, 1, 2, 4, 6, 8 and 10 ml of the dilute standard (7b) and dilute to volume with distilled water. These standards represent concentrations of indican equivalent to 0, 1, 2, 4, 6, 8 and 10 mg per 100 ml sample. Prepare just before use and proceed immediately as follows.

Technic

1. Transfer 1 ml of each calibration standard (7c) to a correspondingly marked 15 ml Pyrex graduated centrifuge tube.
2. To each tube add
    1.5 ml distilled water
    2.5 ml 25% trichloracetic acid (1) and mix
    0.2 ml 1% potassium persulfate (2) and mix
    0.5 ml 1% thymol (3) and mix.
3. Proceed exactly as outlined in the Test Procedure, Steps 8–14 inclusive.
4. Set the zero concentration standard at 100% transmission at a wave length of **540** mμ and record the transmittancy of the 1, 2, 4, 6, 8 and 10 mg per cent standards. The transmittancy of the 10 mg standard should be approximately 31%.
5. Plot the observed values on semi-log graph paper—the points fall on a slight curve which passes through the origin (Calibration Curve I-10).
6. Prepare a calibration table from the T-C values obtained from the plotted curve.

Method I-10b

DETERMINATION IN URINE

SAMPLE

Aliquot of a 24-hour specimen. Preserve with toluene—do not use formaldehyde.

TEST PROCEDURE

Special Apparatus

Graduated centrifuge tubes, Pyrex; 15 ml in 0.1 ml; the 10 ml graduation must be accurately calibrated.

Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.

Reagents

2. **Potassium Persulfate, 1% Aqueous.** Same as for blood.
3. **Thymol, 1% Alcoholic.** Same as for blood.
4. **Ethyl Alcohol, 95%.** Same as for blood.
6. **Acetic Acid, Glacial.** Same as for blood.
8. **Trichloracetic-Hydrochloric Acid Reagent (Meiklejohn-Cohen).** Add 25 gm crystalline trichloracetic acid (CCl_3COOH—J. T. Baker, CP Analyzed) to 100 ml concentrated hydrochloric acid (HCl—Baker & Adamson, A.C.S.); crush the crystals with a footed glass rod and stir until dissolved. Preserve in glass-stoppered bottle. Note: This reagent is not the same as that used for blood.

Technic

Note: For concentrations of indican in excess of 20 mg per 100 ml, use less urine in Step 1 and correct the values obtained from the calibration table for blood accordingly.

1. In a 100 ml volumetric flask place 10 ml thoroughly mixed unfiltered urine and dilute to volume with distilled water.

2. In a 15 ml Pyrex graduated centrifuge tube (sample) place
   5 ml diluted urine
   0.5 ml 1% potassium persulfate (2)
   0.5 ml 1% thymol (3) and mix.
3. In another 15 ml Pyrex graduated centrifuge tube (sample blank) place
   5 ml diluted urine
   0.5 ml 1% potassium persulfate (2)
   0.5 ml 95% ethyl alcohol (4) and mix.
4. To each tube add 5 ml trichloracetic-hydrochloric acid reagent (8), stopper with clean corks and mix thoroughly by inversion.
5. Allow to stand at room temperature for 15 minutes.
6. Place the tubes in vigorously boiling water for 20 minutes—if the aqueous layer is not free of pigment, prolong the boiling period.
7. Proceed exactly as outlined in the Test Procedure for blood, Steps 11–14 inclusive.
8. Measure the transmittancy of the sample against the sample blank set at 100% transmission at a wave length of 540 mμ and multiply the value from the calibration table for blood by 2 to obtain the concentration of indican in mg per 100 ml urine.

Calculation

$$\frac{\text{Volume of 24-hour specimen}}{100} \times \text{Indican in mg per 100 ml} = \text{Mg indican excreted in 24 hours}$$

PROCEDURE FOR USE WITH LINEAR-SCALE PHOTOELECTRIC COLORIMETERS (SHEARD-SANFORD TYPE)

TEST PROCEDURE

Exactly as outlined; set the sample blank at 100 and use a green filter.

CALIBRATION PROCEDURE

Exactly as outlined—use a green filter; plot the observed readings on semi-log graph paper—the points fall on a smooth curve passing through the origin.

PROCEDURE FOR USE WITH LOGARITHMIC-SCALE PHOTOELECTRIC COLORIMETERS (KLETT-SUMMERSON TYPE)

TEST PROCEDURE

Exactly as outlined; set the sample blank at 0 and use a green filter.

CALIBRATION PROCEDURE

Exactly as outlined—use a green filter; plot the observed readings on linear graph paper—the points fall on a smooth curve passing through the origin.

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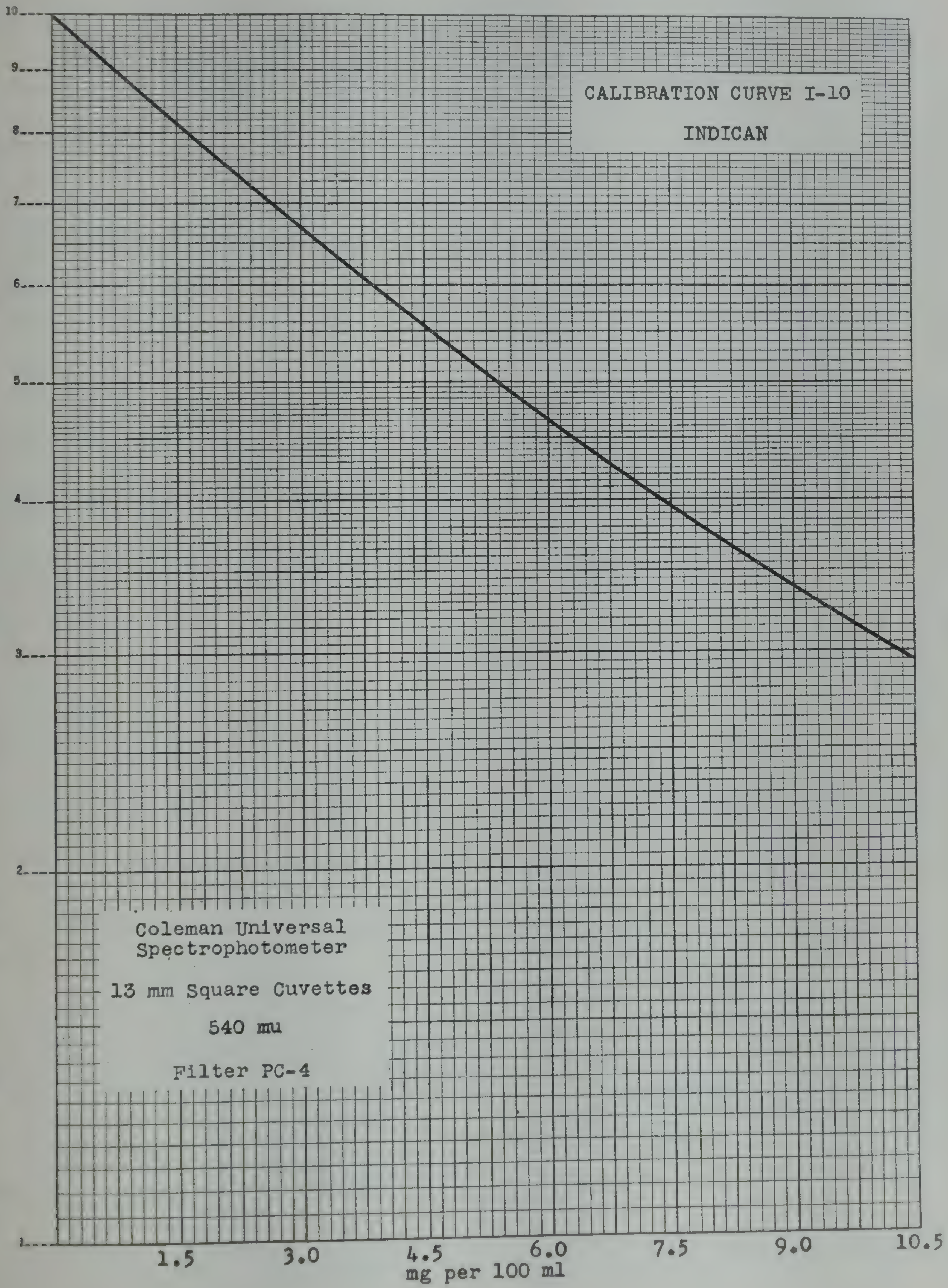
NOTES


CALIBRATION CURVE I-10
INDICAN
Coleman Universal Spectrophotometer
13 mm Square Cuvettes
540 mu
Filter PC-4
10
9
8
7
6
5
4
3
2
1
1.5
3.0
4.5
6.0
7.5
9.0
10.5
mg per 100 ml

Meter Reading	0	1	2	3	4	5	6	7	8	9
0										
10										
20										
30										
40										
50										
60										
70										
80										
90										

INULIN

A spectrophotometric adaptation based on the methods and modifications of van Creveld (1926, 1927), Somogyi (1930), Fujita and Iwatake (1931), Herbert (1938), Corcoran and Page (1939), Alving, Rubin and Miller (1939) and Harrison (1942)

PRINCIPLE

Inulin, in a protein and dextrose-free filtrate, is hydrolyzed by means of hydrochloric acid to levulose which reacts simultaneously with diphenylamine to produce a violet blue color.

DETERMINATION IN PLASMA OR SERUM

SAMPLE

When carrying out the inulin clearance test, three or more samples are necessary—one from blood withdrawn during post-absorptive state prior to the administration of inulin and two or more, depending upon the technic followed, from blood secured at definite intervals after the administration of inulin. Anticoagulant for plasma—potassium oxalate. The plasma or serum must be separated from the red cells as soon as possible. For precise results, dextrose, which reacts slightly with diphenylamine, must be removed by fermentation.

TEST PROCEDURE

Special Apparatus

Wintrobe hematocrit tube and pipette.

Ostwald-Folin pipettes accurately calibrated "to contain" 1 ml.

Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.

Reagents

1. **Yeast Suspension (Alving-Rubin-Miller).** In a glass mortar place 50 gm fresh commercial yeast (Standard Brands) and triturate with a small portion of distilled water to form a thick paste; transfer to a 100 ml mixing cylinder, washing out the mortar with further small portions of distilled water and transferring the washings to the cylinder; dilute to volume and shake thoroughly until completely suspended; transfer the suspension to two 50 ml centrifuge tubes and centrifuge at 2000 rpm for 5 minutes; discard the supernatant fluid; add about 40 ml distilled water to each tube and stir up the sediment thoroughly; again centrifuge and discard the supernatant fluid and repeat the washing until the supernatant fluid is clear and colorless—a total of five washings is necessary; after the final washing, wash out the contents of both tubes into a 100 ml mixing cylinder and dilute to volume with distilled water. Preserve in refrigerator. Stability approximately 2 weeks. Before use the cells should be thoroughly suspended and a portion washed with distilled water. The water content of the yeast cell suspension employed in the analysis is determined as follows: Fill a Wintrobe hematocrit tube to exactly the 100 ml mark with the yeast cell suspension, using a special Wintrobe pipette; centrifuge at high speed to constant volume (at least 30 minutes) and read off the percentage of water in the suspension directly from the scale. This value is necessary in the final calculation.
2. **Acid Cadmium Sulfate Solution (Fujita-Iwatake).** In a 1000 ml volumetric flask place 13 gm crystalline cadmium sulfate ($3CdSO_4 \cdot 8H_2O$—Baker & Adamson, A.C.S.) or 10.59 anhydrous cadmium sulfate ($CdSO_4$—J. T. Baker, A.C.S.) and 63.5 ml 1 N sulfuric acid (a); add approximately 900 ml distilled water and shake until dissolved; dilute to volume with distilled water and filter. Note: If crystals of cadmium sulfate are used they must be clear and show no efflorescence.
    (a) Sulfuric acid, volumetric 1 N; slowly add 30 ml concentrated sulfuric acid (H_2SO_4—Grasselli, A.C.S.) to 1000 ml distilled water and mix; allow to cool; standardize against 1 N alkali using phenolphthalein as indicator.
3. **Sodium Hydroxide, Volumetric 1.1 N.** Prepare by diluting slightly more than the required amount of concentrated carbonate-free sodium hydroxide solution (see Appendix) and titrate against standard 1 N acid using phenolphthalein as indicator; adjust accordingly and re-titrate—20 ml of 1 N acid require 18.2 ml of 1.1 N alkali. Preserve in ceresin-lined bottle.
4. **Diphenylamine Reagent (Harrison).** In a 250 ml mixing cylinder place 100 ml glacial acetic acid (CH_3COOH—Baker & Adamson, A.C.S.); add 3 gm diphenylamine ($(C_6H_5)_2NH$—Eastman #105) and shake until dissolved; add 60 ml concentrated hydrochloric acid (HCl—Baker & Adamson, A.C.S.) and mix by inversion. Preserve in a brown glass-stoppered bottle in refrigerator—a few crystals of diphenylamine may precipitate out of the solution but this is of no consequence. Stability one month.

Technic

Note: For concentrations of inulin in excess of 75 mg per 100 ml, use 0.5 ml sample centrifugate in Steps 6 to 8 and multiply the inulinoid values obtained from the calibration table by 2.

1. In a 15 ml centrifuge tube (sample blank) place
    2 ml plasma or serum from blood withdrawn prior to the administration of inulin
    0.2 ml yeast suspension (1).
2. In another 15 ml centrifuge tube (sample I) place
    2 ml plasma or serum from the first blood specimen withdrawn after the administration of inulin
    0.2 ml yeast suspension (1).
3. In similar 15 ml centrifuge tubes place 2 ml plasma or serum from each additional blood specimen and 0.2 ml yeast suspension (1).
4. Mix the contents of the tubes thoroughly and incubate at 38° C. for 30 minutes.
5. Centrifuge at 2000 rpm for 15 minutes and carefully decant the supernatant fluids into correspondingly marked clean test tubes.
6. In a 25 ml Erlenmeyer flask place
    8 ml acid cadmium sulfate solution (2)
    1 ml sample blank centrifugate from an Ostwald-Folin pipette, thoroughly rinsing out the pipette by drawing in and expelling some of the mixture several times.
7. In another 25 ml Erlenmeyer flask place
    8 ml acid cadmium sulfate solution (2)
    1 ml sample I centrifugate from an Ostwald-Folin pipette in the same manner as before.
8. In similar 25 ml Erlenmeyer flasks place 8 ml acid cadmium sulfate solution (2) and 1 ml centrifugate from each additional sample.
9. Mix thoroughly by a rotatory motion and allow to stand for 1 minute.
10. To each flask add 1 ml 1.1 N sodium hydroxide (3), with constant agitation.
11. Shake vigorously until foam-free and allow to stand for 15 minutes.
12. Centrifuge at 3000 rpm for 5 minutes—the protein-free fluids must be crystal clear.
13. Transfer 0.5 ml of each clear centrifugate to a correspondingly marked 18 × 150 mm Pyrex test tube.
14. To each tube add 1.5 ml distilled water, 4 ml diphenylamine reagent (4) and shake thoroughly until the precipitates dissolve—use a rubber bulb to draw the diphenylamine into the pipette, do not aspirate by mouth.
15. In another 18 × 150 mm Pyrex test tube (reagent blank) place
    2 ml distilled water
    4 ml diphenylamine reagent (4) and mix.
16. Place the tubes in vigorously boiling water for exactly 30 minutes—the tubes should be placed in a rack in the water bath in such a manner that there is free circulation of the water beneath and around the individual tubes.
17. Remove the tubes from the bath and immediately place in cold water and cool to room temperature.
18. Allow the tubes to stand at room temperature for an additional 10 minutes.
19. Measure the transmittancy of the samples and the sample blank against the reagent blank set at 100% transmission at a wave length of 620 mμ and obtain the inulinoid values from the calibration table.

Calculation

Inulinoid value of sample in mg per 100 ml − Inulinoid value of sample blank in mg per 100 ml = Apparent concentration of inulin in mg per 100 ml plasma or serum—result (a)

Correction for dilution due to water in yeast suspension—
(1 + [0.2 × % water in reagent #1]) × result (a) = True concentration of inulin in mg per 100 ml plasma or serum

CALIBRATION PROCEDURE

Special Apparatus

Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.

Reagents

4. **Diphenylamine Reagent (Harrison).** Same as for Test Procedure.
5. **Inulin Standards**
    (a) Inulin stock standard, Herz-Shapiro (1 ml contains 1 mg inulin). Dry recrystallized inulin ($(C_6H_{10}O_5)_x$—Pfanstiehl, C.P.) at 80° C. for one hour and cool in desiccator to constant weight; transfer 100 mg to a 150 ml beaker; add approximately 50 ml distilled water and dissolve with the aid of gentle heat; cool and transfer quantitatively to a 100 ml volumetric flask; add 1 small drop 0.5 N sodium hydroxide and dilute to volume with distilled water. Preserve in refrigerator. Stability two weeks.
    (b) Dilute standard (1 ml contains 0.02 mg inulin). In a 100 ml volumetric flask place 2 ml stock standard (5a) and dilute to volume with distilled water. Prepare just before use.
    (c) Calibration standards. In a series of five accurately calibrated 10 ml volumetric flasks place respectively 0, 2.5, 5.0, 7.5 and 10 ml of the dilute standard (5b) and dilute to volume with distilled water. These standards represent concentrations of inulin equivalent to 0, 20, 40, 60 and 80 mg per 100 ml sample. Prepare just before use and proceed immediately as follows.

Technic

1. Transfer 2 ml of each calibration standard (5c) to a correspondingly marked 18 × 150 mm Pyrex test tube.

2. To each tube add 4 ml diphenylamine reagent (4) and shake thoroughly until the precipitates dissolve—use a rubber bulb to draw the diphenylamine into the pipette, do not aspirate by mouth.
3. Proceed exactly as outlined in the Test Procedure, Steps 16–18 inclusive.
4. Set the zero concentration standard at 100% transmission at a wave length of **620 mμ** and record the transmittancy of the 20, 40, 60 and 80 mg per cent standards. The transmittancy of the 80 mg standard should be approximately 18.5%.
5. Plot the observed values on semi-log graph paper—the points fall on a straight line which passes through the origin (Calibration Curve I-15).
6. Prepare a calibration table from the T-C values obtained from the plotted curve.

Method I-15b

DETERMINATION IN URINE

SAMPLE

First morning fasting specimen and additional specimens as required.

TEST PROCEDURE

Special Apparatus

Wintrobe hematocrit tube and pipette.

Ostwald-Folin pipettes accurately calibrated "to contain" 1 ml.

Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.

Reagents

1. **Yeast Suspension** (**Alving-Rubin-Miller**). Same as for blood.
2. **Acid Cadmium Sulfate Solution** (**Fujita-Iwatake**). Same as for blood.
3. **Sodium Hydroxide, Volumetric 1.1 N.** Same as for blood.
4. **Diphenylamine Reagent** (**Harrison**). Same as for blood.

Technic

Note: For concentrations of inulin in excess of 2000 mg per 100 ml, use 200 ml volumetric flasks in Steps 2 and 3 and multiply the inulinoid values obtained from the calibration table for blood by 50.

1. In a 100 ml volumetric flask (sample blank) place
   50 ml distilled water
   1 ml thoroughly mixed fasting urine specimen from an Ostwald-Folin pipette, thoroughly rinsing out the pipette by drawing in and expelling some of the mixture several times, and dilute to volume with distilled water.
2. In another 100 ml volumetric flask (sample I) place
   50 ml distilled water
   1 ml of the first urine specimen obtained after the administration of inulin, from an Ostwald-Folin pipette in the same manner as before.
3. In similar 100 ml volumetric flasks dilute 1 ml urine from each additional specimen to 100 ml with distilled water in the same manner as before.
4. Transfer 2 ml of each diluted urine to a correspondingly marked 15 ml centrifuge tube and add 0.2 ml yeast suspension (1).
5. Mix the contents of the tubes thoroughly and incubate at 38° C. for 30 minutes.
6. Centrifuge at 2000 rpm for 15 minutes and carefully decant the supernatant fluids into correspondingly marked clean test tubes.
7. In a 25 ml Erlenmeyer flask place
   8 ml acid cadmium sulfate solution (2)
   1 ml sample blank centrifugate from an Ostwald-Folin pipette, thoroughly rinsing out the pipette by drawing in and expelling some of the mixture several times.
8. In another 25 ml Erlenmeyer flask place
   8 ml acid cadmium sulfate solution (2)
   1 ml sample I centrifugate from an Ostwald-Folin pipette in the same manner as before.
9. In similar 25 ml Erlenmyer flasks place 8 ml acid cadmium sulfate solution (2) and 1 ml centrifugate from each additional urine specimen.
10. Mix thoroughly by a rotatory motion and allow to stand for 1 minute.
11. Add 1 ml 1.1 N sodium hydroxide (3), while constantly agitating the tubes.
12. Shake vigorously and allow to stand for 15 minutes.
13. Centrifuge at 3000 for 5 minutes—the protein-free fluids must be crystal clear.
14. Transfer 2 ml of each clear centrifugate to a correspondingly marked 18 × 150 mm Pyrex test tube.
15. To each tube add 4 ml diphenylamine reagent (4) and shake thoroughly until the precipitates dissolve—use a rubber bulb to draw the diphenylamine into the pipette, do not aspirate by mouth.
16. Proceed exactly as outlined in the Test Procedure for blood, Steps 15–18 inclusive.
17. Measure the transmittancy of the samples and the sample blank against the reagent blank set at 100% transmission at a wave length of 620 mμ and multiply the inulinoid values obtained from the calibration table for blood by 25.

Calculation

Inulinoid value of sample in mg per 100 ml − Inulinoid value of sample blank in mg per 100 ml = Apparent concentration of inulin in mg per 100 ml urine − result (a)

Correction for dilution due to water in yeast suspension—
(1 + [0.2 + % water in reagent #1]) × result (a) = True concentration of inulin in mg per 100 ml urine

Note: The Somogyi deproteinizing reagents (1 and 2 under Method I-16) may be used in place of the Fujita-Iwatake solutions (2 and 3). However, when creatinine clearance is determined at the same time, the method for deproteinization as outlined in the test procedure should be followed.

PROCEDURE FOR USE WITH LINEAR-SCALE PHOTOELECTRIC COLORIMETERS (SHEARD-SANFORD TYPE)

TEST PROCEDURE

Exactly as outlined; set the reagent blank at 100 and use a red filter.

CALIBRATION PROCEDURE

Exactly as outlined—use a red filter; plot the observed readings on semi-log graph paper—the points fall on a straight line passing through the origin.

PROCEDURE FOR USE WITH LOGARITHMIC-SCALE PHOTOELECTRIC COLORIMETERS (KLETT-SUMMERSON TYPE)

TEST PROCEDURE

Exactly as outlined; set the reagent blank at 0 and use a red filter.

CALIBRATION PROCEDURE

Exactly as outlined—use a red filter; plot the observed readings on linear graph paper—the points fall on a straight line passing through the origin.

Note: For instruments with cells, cuvettes or tubes requiring a final volume of more than 6 ml, follow the procedure as outlined and add 2 ml diphenylamine reagent (4) just before measurements are made in both the test and calibration procedures.

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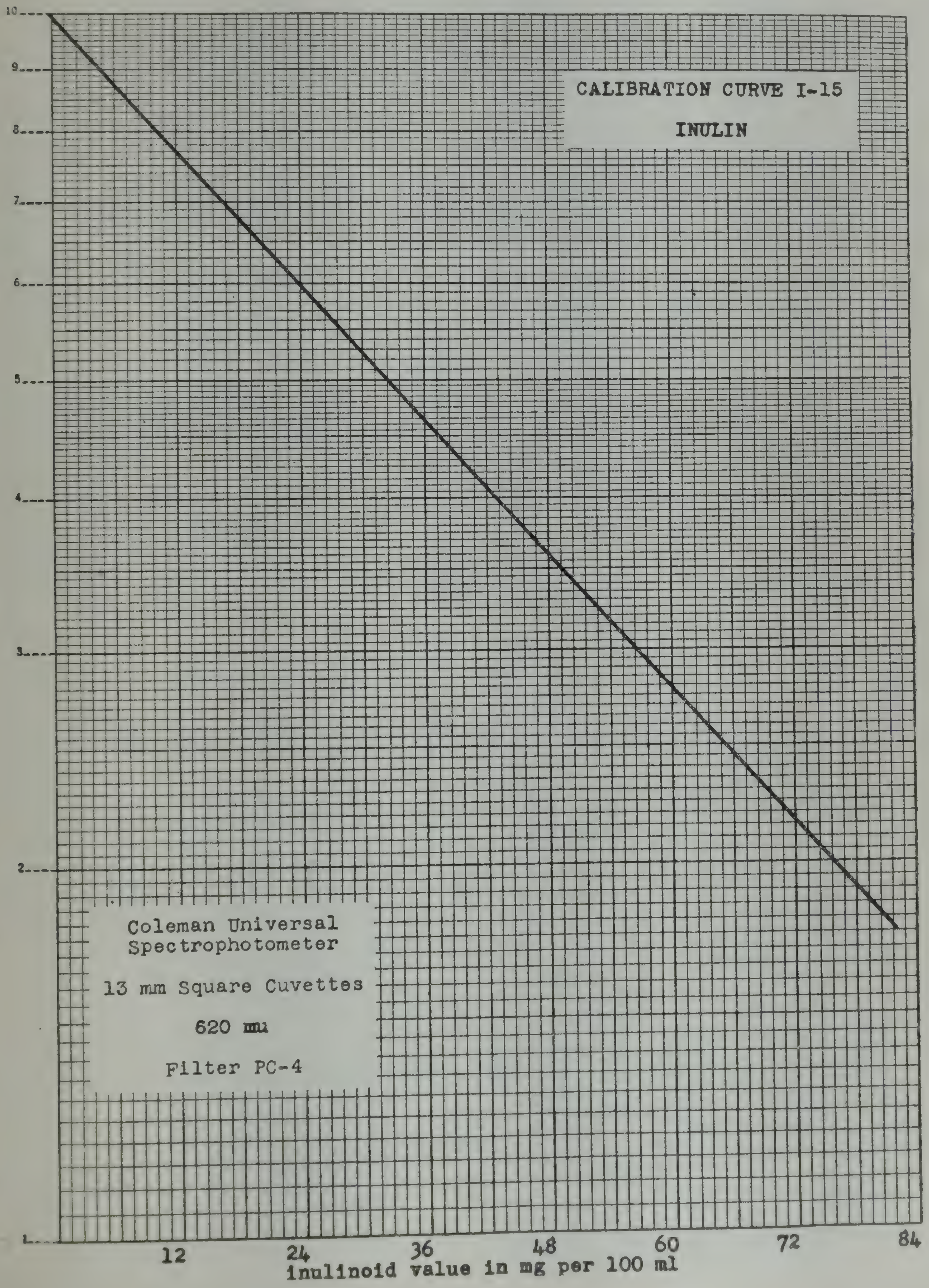
NOTES


CALIBRATION CURVE I-15
INULIN
Coleman Universal Spectrophotometer
13 mm Square Cuvettes
620 mμ
Filter PC-4
10
9
8
7
6
5
4
3
2
1
12
24
36
48
60
72
84
inulinoid value in mg per 100 ml

Meter Reading	0	1	2	3	4	5	6	7	8	9
0										
10										
20										
30										
40										
50										
60										
70										
80										
90										

INULIN

A spectrophotometric adaptation based on the methods and modifications of Somogyi (1930), Roe (1934), Steinitz (1938), Hubbard and Loomis (1942), Herz and Shapiro (1947) and Roe, Epstein and Goldstein (1949)

PRINCIPLE

Inulin is hydrolyzed by means of hydrochloric acid to levulose, which is then treated with resorcinol in the presence of thiourea and acetic acid to develop a red color (Seliwanoff reaction).

DETERMINATION IN PLASMA OR SERUM

SAMPLE

When carrying out the inulin clearance test, three or more samples are necessary—one from blood withdrawn during post-absorptive state prior to the administration of inulin and two or more, depending upon the technic followed, from blood secured at definite intervals after the administration of inulin. Anticoagulant for plasma—potassium oxalate. The plasma or serum must be separated from the red cells as soon as possible. This method is accurate only when the concentration of blood dextrose is within normal limits. For precise work in the presence of increased concentrations of dextrose, Method I-15 must be employed.

TEST PROCEDURE

Special Apparatus

Ostwald-Folin pipettes accurately calibrated "to contain" 1 ml.

Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.

Reagents

1. **Zinc Sulfate, 10% Aqueous (Somogyi).** Dissolve 100 gm crystalline zinc sulfate ($ZnSO_4 \cdot 7H_2O$—J. T. Baker, CP Analyzed) in distilled water; dilute to 1000 ml and filter. Titrate against standardized 0.5 N sodium hydroxide (Reagent #2) with phenolphthalein as follows: place exactly 10 ml of the zinc sulfate in a titration flask and add approximately 100 ml distilled water; add the sodium hydroxide, drop-wise and with constant agitation of the flask, until a pink color persists for at least one minute—10 ml of the sulfate must require 10 ml of the hydroxide—if not, correct accordingly. A slight yellow precipitate settles out on prolonged standing—this may be ignored.
2. **Sodium Hydroxide, Volumetric 0.5 N (Somogyi).** Prepare by diluting slightly more than the required amount of concentrated carbonate-free sodium hydroxide solution (see Appendix) and titrate against standard 1 N acid using phenolphthalein as indicator; adjust accordingly and re-titrate—10 ml of 1 N acid require 20 ml of 0.5 N alkali. Preserve in ceresin-lined bottle.
3. **Resorcinol-Thiourea Reagent (Roe-Epstein-Goldstein).** Dissolve 100 mg white crystalline resorcinol ($C_6H_4(OH)_2$—Mallinckrodt, AR) and 250 mg thiourea (NH_2CSNH_2—Eastman #497) in 100 ml glacial acetic acid (CH_3COOH—Baker & Adamson, A.C.S.). Preserve in a brown glass bottle.
4. **Hydrochloric Acid, 30% Aqueous.** To 20 ml distilled water slowly add 100 ml concentrated hydrochloric acid (HCl—Baker & Adamson, A.C.S.) and mix. Preserve in glass-stoppered bottle.

Technic

Note: For concentrations of inulin in excess of 75 mg per 100 ml use 1 ml filtrate and 1 ml distilled water in Steps 7 to 9 and multiply the inulinoid values obtained from the calibration table by 2. Exact measurements and timing are essential.

1. In a 50 ml Erlenmeyer flask (sample blank) place
   15 ml distilled water
   1 ml plasma or serum from blood withdrawn prior to the administration of inulin, from an Ostwald-Folin pipette, thoroughly rinsing out the pipette by drawing in and expelling some of the mixture several times.
2. In another 50 ml Erlenmeyer flask (sample I) place
   15 ml distilled water
   1 ml plasma or serum from the first blood specimen withdrawn after the administration of inulin, from an Ostwald-Folin pipette in the same manner as before.
3. In similar 50 ml Erlenmeyer flasks place 15 ml distilled water and 1 ml plasma or serum from each additional blood specimen.
4. After laking is complete add 2 ml 10% zinc sulfate (1) to each flask and mix thoroughly by a rotatory motion.
5. Add 2 ml 0.5 N sodium hydroxide (2), shake vigorously until foam-free and allow to stand for 15 minutes.
6. Filter the contents of all flasks through Schleicher and Schuell #576 papers into correspondingly marked test tubes, covering the funnels with watch crystals during filtration.
7. In an 18 × 150 mm Pyrex test tube place 2 ml sample blank filtrate.
8. In a second 18 × 150 mm Pyrex test tube place 2 ml sample I filtrate.
9. In similar 18 × 150 mm Pyrex test tubes place 2 ml filtrate from each additional sample.
10. In another 18 × 150 mm Pyrex test tube (reagent blank) place 2 ml distilled water.

11. To each tube add 1 ml resorcinol-thiourea reagent (3) and mix.
12. Add 7 ml 30% hydrochloric acid (4) and mix thoroughly by gentle shaking.
13. Place tubes in water bath at 80° C. for exactly 10 minutes—the level of the water in the bath must be above the level of the fluid in the tubes.
14. Remove tubes from bath and cool under running tap water to room temperature, keeping the tubes shaded during the cooling period.
15. Immediately measure the transmittancy of the samples and the sample blank against the reagent blank set at 100% transmission at a wave length of 520 mμ and obtain the inulinoid values from the calibration table.

Calculation

Inulinoid value of sample in mg per 100 ml − Inulinoid value of sample blank in mg per 100 ml = Concentration of inulin in mg per 100 ml plasma or serum

CALIBRATION PROCEDURE

Special Apparatus

Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.

Reagents

3. **Resorcinol-Thiourea Reagent (Roe-Epstein-Goldstein).** Same as for Test Procedure.
4. **Hydrochloric Acid, 30% Aqueous.** Same as for Test Procedure.
5. **Inulin Standards**
    (a) Inulin stock standard, Herz-Shapiro (1 ml contains 1 mg inulin). Dry recrystallized inulin (($(C_6H_{10}O_5)_x$—Pfanstiehl, C.P.) at 80° C. for one hour and cool in desiccator to constant weight; transfer 100 mg to a 150 ml beaker, add approximately 50 ml distilled water and dissolve with the aid of gentle heat; cool and transfer quantitatively to a 100 ml volumetric flask; add 1 small drop 0.5 N sodium hydroxide and dilute to volume with distilled water. Preserve in refrigerator. Stability two weeks.
    (b) Dilute standard (1 ml contains 0.04 mg inulin). In a 100 ml volumetric flask place 4 ml stock standard (5a) and dilute to volume with distilled water. Prepare just before use.
    (c) Calibration standards. In a series of five accurately calibrated 10 ml volumetric flasks place respectively 0, 2.5, 5, 7.5 and 10 ml of the dilute standard (5b) and dilute to volume with distilled water. These standards represent concentrations of inulin equivalent to 0, 20, 40, 60 and 80 mg per 100 ml sample. Prepare just before use and proceed immediately as follows.

Technic

1. Transfer 2 ml of each calibration standard (5c) to a correspondingly marked 18 × 150 mm Pyrex test tube.
2. Proceed exactly as outlined in the Test Procedure, Steps 11–14 inclusive.
3. Immediately set the zero concentration standard at 100% transmission at a wave length of 520 mμ and record the transmittancy of the 20, 40, 60 and 80 mg per cent standards. The transmittancy of the 80 mg standard should be approximately 44%.
4. Plot the observed values on semi-log graph paper—the points fall on a straight line which passes through the origin (Calibration Curve I-16).
5. Prepare a calibration table from the T-C values obtained from the plotted curve.

Method I-16b

DETERMINATION IN URINE

SAMPLE

First morning fasting specimen and additional specimens as required. This method is accurate only when the concentration of urine dextrose is less than 200 mg per 100 ml. For precise work in the presence of greater concentrations of dextrose, Method I-15 must be employed.

TEST PROCEDURE

Special Apparatus

As listed under the Test Procedure for blood.

Reagents

1. **Zinc Sulfate, 10% Aqueous.** Same as for blood.
2. **Sodium Hydroxide, Volumetric 0.5 N.** Same as for blood.
3. **Resorcinol-Thiourea Reagent (Roe-Epstein-Goldstein).** Same as for blood.
4. **Hydrochloric Acid, 30% Aqueous.** Same as for blood.

Technic

1. In a 50 ml Erlenmeyer flask (sample blank) place
    15 ml distilled water
    1 ml thoroughly mixed fasting urine specimen from an Ostwald-Folin pipette, thoroughly rinsing out the pipette by drawing in and expelling some of the mixture several times.

2. In another 50 ml Erlenmeyer flask (sample I) place
   15 ml distilled water
   1 ml of the first urine specimen obtained after the administration of inulin, from an Ostwald-Folin pipette in the same manner as before.
3. In similar 50 ml Erlenmeyer flasks place 15 ml distilled water and 1 ml urine from each additional specimen.
4. To each flask add 2 ml 10% zinc sulfate (1) and mix thoroughly by a rotatory motion.
5. Add 2 ml 0.5 N sodium hydroxide (2), shake vigorously until foam-free and allow to stand for 15 minutes.
6. Filter the contents of all flasks through Schleicher and Schuell #576 papers into correspondingly marked test tubes, covering the funnels with watch crystals during filtration.
7. Dilute the filtrates with distilled water so that the inulin content is less than 4 mg per 100 ml.
8. In an 18 × 150 mm Pyrex test tube place 2 ml diluted sample blank filtrate.
9. In a second 18 × 150 mm Pyrex test tube place 2 ml diluted sample I filtrate.
10. In similar 18 × 150 mm Pyrex test tubes place 2 ml diluted filtrate from each additional sample.
11. In another 18 × 150 mm Pyrex test tube (reagent blank) place 2 ml distilled water.
12. Proceed exactly as outlined in the Test Procedure for blood, Steps 11–14 inclusive.
13. Immediately measure the transmittancy of the samples and the sample blank against the reagent blank set at 100% transmission at a wave length of 520 mμ and multiply the inulinoid values from the calibration table for blood by the proper dilution factor (from Step 7).

Calculation

Inulinoid value of sample in mg per 100 ml − Inulinoid value of sample blank in mg per 100 ml = Concentration of inulin in mg per 100 ml urine

PROCEDURE FOR USE WITH LINEAR-SCALE PHOTOELECTRIC COLORIMETERS (SHEARD-SANFORD TYPE)

TEST PROCEDURE

Exactly as outlined; set the reagent blank at 100 and use a green filter.

CALIBRATION PROCEDURE

Exactly as outlined—use a green filter; plot the observed readings on semi-log graph paper—the points fall on a straight line passing through the origin.

PROCEDURE FOR USE WITH LOGARITHMIC-SCALE PHOTOELECTRIC COLORIMETERS (KLETT-SUMMERSON TYPE)

TEST PROCEDURE

Exactly as outlined; set the reagent blank at 0 and use a green filter.

CALIBRATION PROCEDURE

Exactly as outlined—use a green filter; plot the observed readings on linear graph paper—the points fall on a straight line passing through the origin.

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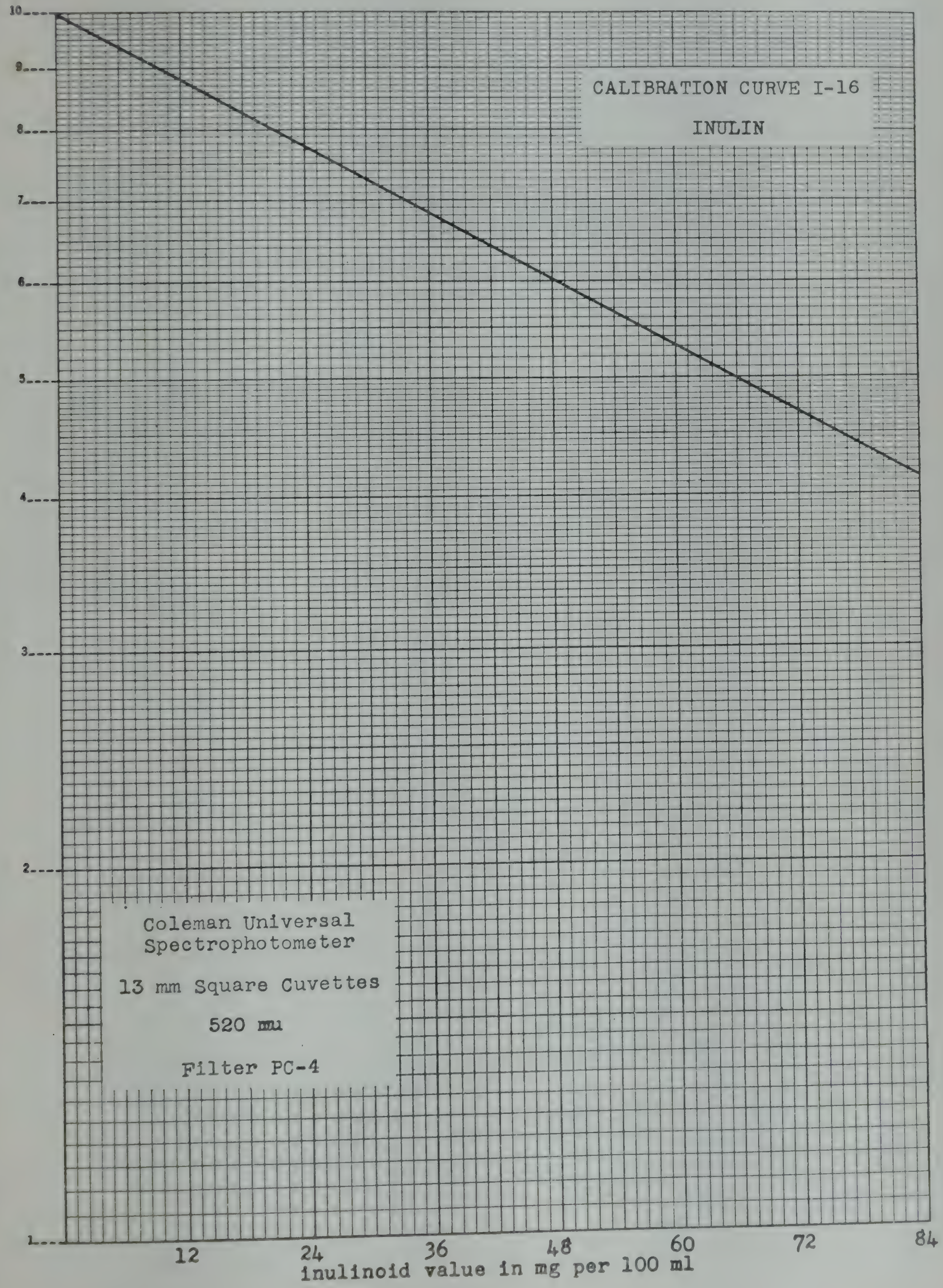
NOTES


CALIBRATION CURVE I-16
INULIN
Coleman Universal Spectrophotometer
13 mm Square Cuvettes
520 mμ
Filter PC-4
10
9
8
7
6
5
4
3
2
1
12
24
36
48
60
72
84
inulinoid value in mg per 100 ml

Meter Reading	0	1	2	3	4	5	6	7	8	9
0										
10										
20										
30										
40										
50										
60										
70										
80										
90										

IODINE, TOTAL

A spectrophotometric adaptation based on the methods and modifications of Leipert (1933), Sandell and Kolthoff (1937); Stevens (1937); Chaney (1940); Salter (1940); Lein (1941); Man, Smirnow, Gildea and Peters (1942); Talbot, Butler, Saltzman and Rodriguez (1944); Taurog and Chaikoff (1946) and Barker (1948)

PRINCIPLE

The sample, without any previous deproteinization, is digested directly with a sulfuric-chromic acid mixture to convert the iodine to non-volatile iodic acid. Free iodine, which is liberated by phosphorous acid, is distilled off and absorbed in sodium sulfite solution, the excess sulfite being destroyed by sulfuric acid. The reducing agent-free distillate is then treated with arsenious acid and ceric cerium, and since the oxidation-reduction reaction between these two substances is catalyzed by iodine, the measurement of the amount of reduction of the cerium (decrease of the yellow color) in a definite time interval is a measure of the iodine content of the sample.

DETERMINATION IN WHOLE BLOOD, PLASMA OR SERUM

SAMPLE

Oxalated or heparinized whole blood or plasma, or serum; plasma or serum analyses preferable. Ordinary potassium oxalate should not be used as an anticoagulant—use only the specially recrystallized salt (Reagent #1). Due to the extreme sensitivity of this determination, every possible precaution should be taken to avoid contamination. Use no iodine for sterilization purposes and have no iodine (even if in a stoppered bottle) in the immediate vicinity either while withdrawing the blood or when performing the analysis. For precise results, tincture of iodine or other volatile forms of iodine should not even be in the same room.

TEST PROCEDURE

Special Apparatus

Ostwald-Folin pipettes accurately calibrated "to deliver" 2 ml.

Chaney-Riggs distillation apparatus (Fig. I-19) consisting of a 250 ml round-bottom flask (a) with two necks which serves for both digestion and distillation; during digestion, the flask only is used; during distillation, the digested solution in the flask is heated, and as the steam forms, it passes up column (b), past the baffle plate, then to the right and downward through the trap (c), then to the left and upward to the chamber (d); it then passes by the second baffle plate and into the condenser (e); the condensed water drips down and is returned by tube (f) to column (b) from which it drips back into the distillation flask (a). The dropping funnel is inserted in the eccentric neck of the flask and is used for adding the phosphorous acid. Note: All connections are standard taper glass joints.

Graduated test tubes—25 ml in 0.5 ml; the 25 ml graduation must be accurately calibrated.

Pipette with fine tip and small orifice, the volume between two graduation marks being exactly 0.5 ml.

Cuvettes accurately graduated at 9.5 ml.

Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.

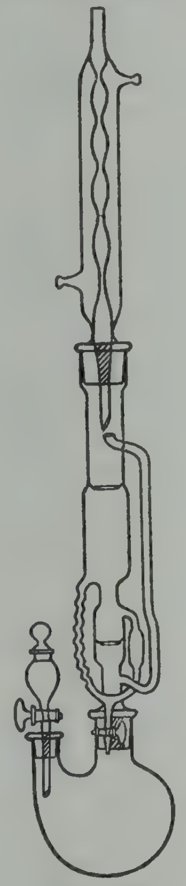


Fig. I-19

Note: All apparatus, including the cuvettes, must be scrupulously clean—wash with hot dichromate-sulfuric acid solution (see Appendix), rinse thoroughly with distilled water and finally with iodine-free distilled water (Reagent #2) and oven dry before use. The central portion of the distillation apparatus must always be cleaned in this manner between runs.

Stopcocks must be lubricated with iodine-free distilled water only—grease must not be used. Precautions should be taken to prevent freezing of the stopcocks due to drying.

Reagents

1. **Potassium Oxalate, Iodine-Free.** Prepare a saturated aqueous solution of potassium oxalate ($K_2C_2O_4 \cdot H_2O$—J. T. Baker, A.C.S.) in iodine-free distilled water (2) and recrystallize by adding 95% iodine-free ethyl alcohol (a) until precipitation is complete; filter on a sintered glass funnel, suck as dry as possible and dry in desiccator.
   (a) Ethyl alcohol, 95%, iodine-free; redistil 95% ethyl alcohol (C_2H_5OH—U. S. Industrial Chemicals, U.S.P.) from solid potassium hydroxide (KOH—Baker & Adamson, A.C.S.).
2. **Water, Distilled, Iodine-Free.** Redistil distilled water from potassium hydroxide (35 gm solid potassium hydroxide for each 100 ml distilled water) from an all-glass Pyrex distillation apparatus, discarding the distillate until neutral to methyl red. Preserve in Pyrex glass-stoppered bottle. Use for the preparation of all reagents and standards and where indicated in the test procedure.
3. **Sulfuric Acid, 70% by Weight.** In a 2000 ml Pyrex flask place 600 ml iodine-free distilled water (2) and slowly add 780 ml concentrated sulfuric acid (H_2SO_4—Grasselli, A.C.S.), while cooling the flask under running tap water; mix thoroughly and allow to cool, keeping the flask covered while cooling. Preserve in Pyrex glass-stoppered bottle.

Note: The concentrated acid specified has generally been found to be satisfactory; other acids should be purified by distilling from an all-glass retort or by adding 10 ml concentrated hydrochloric acid to 2000 ml concentrated sulfuric acid and boiling for 2 hours under a hood.

4. **Chromic Acid, 60% Aqueous.** Dissolve 600 gm chromium trioxide (CrO_3—Mutual Chemical) in iodine-free distilled water (2) and dilute to 1000 ml. Preserve in Pyrex glass-stoppered bottle. Note: The iodine content of chromium trioxide varies considerably, many reagent grade samples showing a higher iodine content than technical grade material; new lots of the trioxide, even though from the same manufacturer, should always be checked for their iodine content.
5. **Sodium Sulfite, 10% Aqueous.** Just prior to use dissolve 1 gm anhydrous sodium sulfite (Na_2SO_3—Mallinckrodt, A.C.S.) in 10 ml iodine-free distilled water (2).
6. **Phosphorous Acid, 50% Aqueous.** In a 1000 ml Pyrex flask place 250 gm crystalline phosphorous acid (H_3PO_3—Baker & Adamson, Reagent); add approximately 300 ml iodine-free distilled water (2) and shake until dissolved; boil for 30 minutes, adding iodine-free distilled water frequently to keep the volume between 400–500 ml; allow to cool and dilute to 500 ml with iodine-free distilled water. Preserve in Pyrex glass-stoppered bottle.
7. **Sulfuric Acid, Volumetric 1 N.** Slowly add 30 ml concentrated sulfuric acid (H_2SO_4—Grasselli, A.C.S.) to 1000 ml iodine-free distilled water (2) and mix; allow to cool and standardize against 1 N alkali using phenolphthalein as indicator. Preserve in Pyrex glass-stoppered bottle.
8. **Arsenious Acid Reagent (Barker).** In a 400 ml beaker place 50 ml 1 N sodium hydroxide (a); add 3.71 gm dry arsenious acid (As_2O_3—Merck, A.C.S., Primary Standard) and stir until dissolved; add 200 ml iodine-free distilled water (2) and mix; add sufficient 70% sulfuric acid (3) until neutral to litmus—about 2.5 ml are required; add, with constant stirring, an additional 54 ml 70% sulfuric acid and quantitatively transfer to a 500 ml volumetric flask; dilute to volume with iodine-free distilled water, allow to cool and re-adjust volume; add 3.125 gm iodine-free sodium chloride (NaCl—Mallinckrodt, A.C.S.) and shake until dissolved. Preserve in Pyrex glass-stoppered bottle.
    (a) Sodium hydroxide, volumetric 1 N; prepare by diluting slightly more than the required amount of concentrated carbonate-free sodium hydroxide solution (see Appendix) and standardize against 1 N acid using phenolphthalein as indicator. Preserve in Pyrex glass-stoppered bottle.
9. **Ceric Sulfate Reagent (Barker).** In a 1000 ml beaker place 500 ml 3.5 N sulfuric acid (a); add 12 gm dry ceric ammonium sulfate ($(NH_4)_4Ce(SO_4)_4 \cdot 2H_2O$—G. Frederick Smith, Reagent) and stir thoroughly. The solution is turbid but, with occasional stirring, clears within 30 minutes. Preserve in Pyrex glass-stoppered bottle.
    (a) Sulfuric acid, volumetric 3.5 N; slowly add 105 ml concentrated sulfuric acid (H_2SO_4—Grasselli, A.C.S.) to 900 ml iodine-free distilled water (2); mix and allow to cool; dilute 20 ml of this approximately 3.5 N solution to 100 ml; titrate against standard 1 N alkali using phenolphthalein as indicator; adjust accordingly and re-titrate—20 ml of a 1:5 dilution of 3.5 N acid require 14 ml of 1 N alkali. Preserve in Pyrex glass-stoppered bottle.
10. **Sulfuric Acid, Volumetric 0.04 N.** Dilute 4 ml 1 N sulfuric acid (7) to 100 ml with iodine-free distilled water (2) and mix. This solution is used only for excessive concentrations of iodine—see note under technic.

Technic

Note: For concentrations of total iodine less than 2.5 gamma per 100 ml, use a 30 minute reduction period in Steps 27 to 29 and obtain the values directly from the calibration table prepared from curve I-19B; for concentrations in excess of 25 gamma per 100 ml, use a 2.5 ml aliquot and 2.5 ml 0.04 N sulfuric acid (10) in Step 20 and multiply the values obtained from the calibration table prepared from curve I-19A by 2.

1. In a digestion flask (sample) place
    25 ml 70% sulfuric acid (3)
    2 ml sample from an Ostwald-Folin pipette, added drop by drop, while gently agitating the flask
    5 ml iodine-free distilled water (2).
2. In another digestion flask (reagent blank) place
    25 ml 70% sulfuric acid (3)
    5 ml iodine-free distilled water (2).

Note: From this point on, both sample and reagent blank are treated in an identical manner.

3. While gently shaking the flask, add 3 ml 60% chromic acid (4) and a few glass beads; continue shaking until foaming has ceased.
4. Heat over the small flame of a micro burner until bubbling due to the decomposition of the excess chromic acid ceases and sulfuric acid fumes fill the flask (about 1–2 minutes after initial fuming); heat slowly with constant shaking at the start—severe bumping may occur if the acid mixture becomes too hot (over 200° C.); carry out this step under a fume hood.
5. Allow to cool to less than 100° C., carefully add 15 ml iodine-free distilled water (2), washing down the sides of the flask at the same time and mix the contents by gentle agitation.
6. Repeat Step 4, keeping the temperature at approximately 145° C.—do not prolong the heating period beyond the definite appearance of fumes.
7. Allow to cool to less than 100° C., add a few additional glass beads and 25 ml iodine-free distilled water (2) and dissolve the precipitated chromic acid by rotating the flask. Note: This should be done just prior to distillation to utilize the heat generated.

8. Attach the flask to the distillation apparatus and start heating over micro burner.
9. Through the upper opening of the trap add sufficient iodine-free distilled water (2) to fill the region of attachment of the stopcock.
10. Add 0.5 ml 10% sodium sulfite (5), allowing to drain down the walls of the bulge above the trap—most of the solution collects in the lower portion of the trap and soon after distillation has begun, the condensed water vapor completely closes the trap.
11. Connect the water-cooled condenser in such a manner that the condensate enters the trap; allow to remain in this position for about 30 seconds and then adjust the condenser so that the condensate returns to the flask.
12. Insert the dropping funnel in the eccentric neck of the flask and place 5 ml 50% phosphorous acid (6) in the funnel.
13. Boil until water vapor enters the condenser and starts to drip into the return tube and then slowly blow the phosphorous acid in the funnel into the flask by gentle pressure—it is essential that the trap be sealed by fluid before the phosphorous acid is added.
14. Continue the distillation at 140–145° C. for 30 minutes after reduction is completed (digest becomes colorless). Note: Shorter distillation periods may be used but correction must be made for incomplete recovery.
15. Terminate the distillation by turning off the flame of the micro burner and immediately draining the contents of the trap through the stopcock into a 25 ml graduated test tube.
16. Raise the condenser until clear of the apparatus and rinse down the walls of the trap with five successive 2.5 ml portions iodine-free distilled water (2), adding the washings to the 25 ml test tube.
17. Add 1 ml 1 N sulfuric acid (7) to the combined distillate and washings.
18. Place the tube in boiling water and aerate at about 2.5 liters per minute until the sulfur dioxide is completely blown off—this may be done by using a manifold with outlets for three tubes; the outlets, which are finely drawn out and reach nearly to the bottom of each tube, are provided with screw-clamp controls to prevent excessive air pressure.

Note: If the sample, standard and reagent blank are carried through consecutively to Step 18, they may be aerated simultaneously.

19. Allow to cool, rinse off the manifold outlet, dilute to 25 ml with iodine-free distilled water (2) and mix thoroughly.
20. Transfer 5 ml aliquots of both sample and reagent blank distillates to correspondingly marked graduated cuvettes.
21. In a third graduated cuvette (reference blank) place 5 ml iodine-free distilled water (2).
22. To each cuvette add 0.4 ml arsenious acid reagent (8) and mix.
23. Dilute the contents of each cuvette to exactly 9.5 ml with iodine-free distilled water (2) and mix.
24. To the reference blank add exactly 0.5 ml ceric sulfate reagent (9) from a special pipette, mix, NOTE THE TIME AND IMMEDIATELY place in water bath at 37° C. (±0.1° C.)—the level of the fluid in the cuvette must be below the level of the water in the bath.

Note: The measurement of the ceric sulfate reagent must be extremely exact—the accuracy of the result is dependent upon it.

25. After an interval of 1 minute, add exactly 0.5 ml ceric sulfate reagent (9) from a special pipette to the reagent blank, mix, NOTE THE TIME AND IMMEDIATELY place in water bath at 37° C.
26. After another interval of 1 minute, add exactly 0.5 ml ceric sulfate reagent (9) from a special pipette to the sample, mix, NOTE THE TIME AND IMMEDIATELY place in water bath at 37° C.
27. EXACTLY 15 minutes from the time noted in Step 24, remove the reference blank from the water bath, wipe dry, place in spectrophotometer and set at *20%* transmission at a wave length of 420 mμ—this must be carried out as rapidly as possible so that the elapsed time between the removal from the bath and the instrument setting is less than 1 minute.
28. EXACTLY 15 minutes from the time noted in Step 25, remove the reagent blank from the water bath, wipe dry and measure the transmittancy against the reference blank which has already been set at *20%* transmission and record as result (a)—the elapsed time between the removal from the bath and the transmittancy measurement must be less than 1 minute.
29. EXACTLY 15 minutes from the time noted in Step 26, remove the sample from the water bath, wipe dry and measure the transmittancy against the reference blank which has already been set at *20%* transmission and record as result (b).

Note: In Steps 24–28 inclusive, a 1 minute interval is used between the preparation of the respective tubes prior to incubation, the same time interval being allowed for the transmittancy measurement of each tube after incubation. This interval may be shortened or lengthened according to the analyst's proficiency. Either a fifteen or thirty minute reduction period may be used, according to the iodine content of the sample. The success of the entire procedure is dependent upon the maintenance of a rigid time schedule in these steps.

30. Obtain the values for the recorded transmittancy readings from the calibration table (for the 15 minute reduction period use the calibration table prepared from Calibration Curve I-19A; for the 30 minute period use the table prepared from Calibration Curve I-19B) and calculate the concentration of total iodine as follows.

Calculation

Value for sample in gamma per 100 ml (result (b)) — Value for reagent blank in gamma per 100 ml result (a)) = Total iodine in gamma per 100 ml sample

CALIBRATION PROCEDURE

Note: The calibration curve should be used for comparative purposes only—a standard should always be run with each sample or series of samples. Even though the details of the technic are under such perfect control that reliance may be placed on a calibration curve, it must nevertheless be checked every time a new lot of any reagent is prepared.

Special Apparatus

Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.

Cuvettes accurately graduated at 9.5 ml.

Pipette with fine tip and small orifice, the volume between two graduation marks being exactly 0.5 ml.

Reagents

2. **Water, Distilled, Iodine-Free.** Same as for Test Procedure.
8. **Arsenious Acid Reagent (Barker).** Same as for Test Procedure.
9. **Ceric Sulfate Reagent (Barker).** Same as for Test Procedure.
10. **Sulfuric Acid, Volumetric 0.04 N.** Same as for Test Procedure.
11. **Iodine Standards**
    (a) Iodine stock standard (1 ml contains 100 gamma iodine). Dry crystalline potassium iodide (KI—J. T. Baker, Special, Low in Iodates) in a desiccator to constant weight; transfer 130.8 mg to a 1000 ml volumetric flask; dissolve in iodine-free distilled water (2) and dilute to volume. Preserve in Pyrex brown glass-stoppered bottle away from direct light. Prepare fresh monthly.
    (b) Dilute standard (1 ml contains 1 gamma iodine). In a 100 ml volumetric flask place 1 ml stock standard (11a) and dilute to volume with iodine-free distilled water (2). Preserve in Pyrex brown glass-stoppered bottle away from direct light. Prepare fresh weekly.
    (c) Calibration standards. In a series of eight accurately calibrated 100 ml volumetric flasks place respectively 0, 0.5, 1, 2, 4, 6, 8 and 10 ml of the dilute standard (11b) and dilute to volume with iodine-free distilled water (2). These standards represent concentrations of iodine equivalent to 0, 1.25, 2.5, 5, 10, 15, 20 and 25 gamma per 100 ml sample. Prepare just before use and proceed immediately as follows.

Technic

1. Transfer 1 ml of each calibration standard (11c) to a correspondingly marked graduated cuvette.
2. To each cuvette add 5 ml 0.04 N sulfuric acid (10) and mix.
3. Add 0.4 ml arsenious acid reagent (8) and mix.
4. Dilute to exactly 9.5 ml with iodine-free distilled water (2) and mix.
5. To the zero concentration standard add exactly 0.5 ml ceric sulfate reagent (9) from a special pipette, mix, NOTE THE TIME AND IMMEDIATELY place in water bath at 37° C. (±0.1° C.)—the level of the fluid in the cuvette must be below the level of the water in the bath.

Note: The measurement of the ceric sulfate reagent must be extremely exact—the accuracy of the calibration is dependent upon it.

6. After an interval of 1 minute, add exactly 0.5 ml ceric sulfate reagent (9) from a special pipette to the first calibration standard, mix, NOTE THE TIME AND IMMEDIATELY place in water bath at 37° C.
7. After 1 minute intervals, treat the subsequent calibration standards in an identical manner.
8. EXACTLY 15 minutes from the time noted in Step 5, remove the zero concentration standard from the water bath, wipe dry, place in spectrophotometer and set at *20%* transmission at a wave length of 420 mμ—this must be carried out as rapidly as possible so that the elapsed time between the removal from the bath and the instrument setting is less than 1 minute.
9. EXACTLY 15 minutes from the time noted in Step 6, remove the first calibration standard from the water bath, wipe dry, measure the transmittancy against the zero concentration standard which has already been set at *20%* transmission and record; immediately replace in the water bath—the elapsed time between the removal of the cuvette from the bath and its replacement must be less than 1 minute.
10. After 1 minute intervals, measure the transmittancy of each subsequent calibration standard in a similar manner, immediately replacing each cuvette in the water bath after the transmittancy measurement has been made (see note following Step 29 in the Test Procedure)—the recorded measurements for the 15 minute incubation period are used for the preparation of Calibration Curve I-19A.
11. EXACTLY 30 minutes from the time noted in Steps 5, 6 and 7, repeat the transmittancy measurements—the recorded measurements for the 30 minute incubation period are used for the preparation of Calibration Curve I-19B.
12. Plot the observed values for the 15 minute reduction period on semi-log graph paper—the points fall on a straight line which passes through the

transmission axis at 20% transmission and slopes upward (Calibration Curve I-19A). The transmittancy of the 25 gamma standard should be approximately 85%.

13. Plot the observed values for the 30 minute reduction period on semi-log graph paper (only the values for the first four calibration standards can be plotted)—the points fall on a straight line which passes through the transmission axis at 20% transmission and slopes upward (Calibration Curve I-19B). The transmittancy of the 10 gamma standard should be approximately 64%.
14. Prepare calibration tables from the T-C values obtained from each plotted curve.

Method I-19b

DETERMINATION IN URINE

SAMPLE

Aliquot of a 24-hour specimen. Collect in Pyrex glass-stoppered bottles, taking due precautions to avoid contamination by iodine.

TEST PROCEDURE

Special Apparatus

As listed under the Test Procedure for blood.

Reagents

2. **Water, Distilled, Iodine-Free.** Same as for blood.
3. **Sulfuric Acid, 70% by Weight.** Same as for blood.
4. **Chromic Acid, 60% Aqueous.** Same as for blood.
5. **Sodium Sulfite, 10% Aqueous.** Same as for blood.
6. **Phosphorous Acid, 50% Aqueous.** Same as for blood.
7. **Sulfuric Acid, Volumetric 1 N.** Same as for blood.
8. **Arsenious Acid Reagent (Barker).** Same as for blood.
9. **Ceric Sulfate Reagent (Barker).** Same as for blood.
10. **Sulfuric Acid, Volumetric 0.04 N.** Same as for blood.

Technic

Note: For concentrations of total iodine in excess of 25 gamma per 100 ml, use a 2.5 ml aliquot and 2.5 ml 0.04 N sulfuric acid (10) in Step 20 and multiply the values obtained from the calibration table for blood prepared from curve I-19A by 2.

1. In a digestion flask (sample) place
    25 ml 70% sulfuric acid (3)
    2 ml thoroughly mixed unfiltered urine from an Ostwald-Folin pipette, added drop by drop, while gently agitating the flask
    5 ml iodine-free distilled water (2).
2. Proceed exactly as outlined in the Test Procedure for blood, Steps 2–30 inclusive.

PROCEDURE FOR USE WITH LINEAR-SCALE PHOTOELECTRIC COLORIMETERS (SHEARD-SANFORD TYPE)

TEST PROCEDURE

Exactly as outlined; set the reference blank at 20 and use a blue filter.

CALIBRATION PROCEDURE

Exactly as outlined—use a blue filter; plot the observed readings on semi-log graph paper—the points fall on straight lines which intersect the transmission axis at 20% and slope upward.

PROCEDURE FOR USE WITH LOGARITHMIC-SCALE PHOTOELECTRIC COLORIMETERS (KLETT-SUMMERSON TYPE)

TEST PROCEDURE

Exactly as outlined; set the reference blank at 300 and use a blue filter.

CALIBRATION PROCEDURE

Exactly as outlined—use a blue filter; plot the observed readings on linear graph paper—the points fall on practically straight lines which intersect the instrument reading axis at 300 and slope downward.

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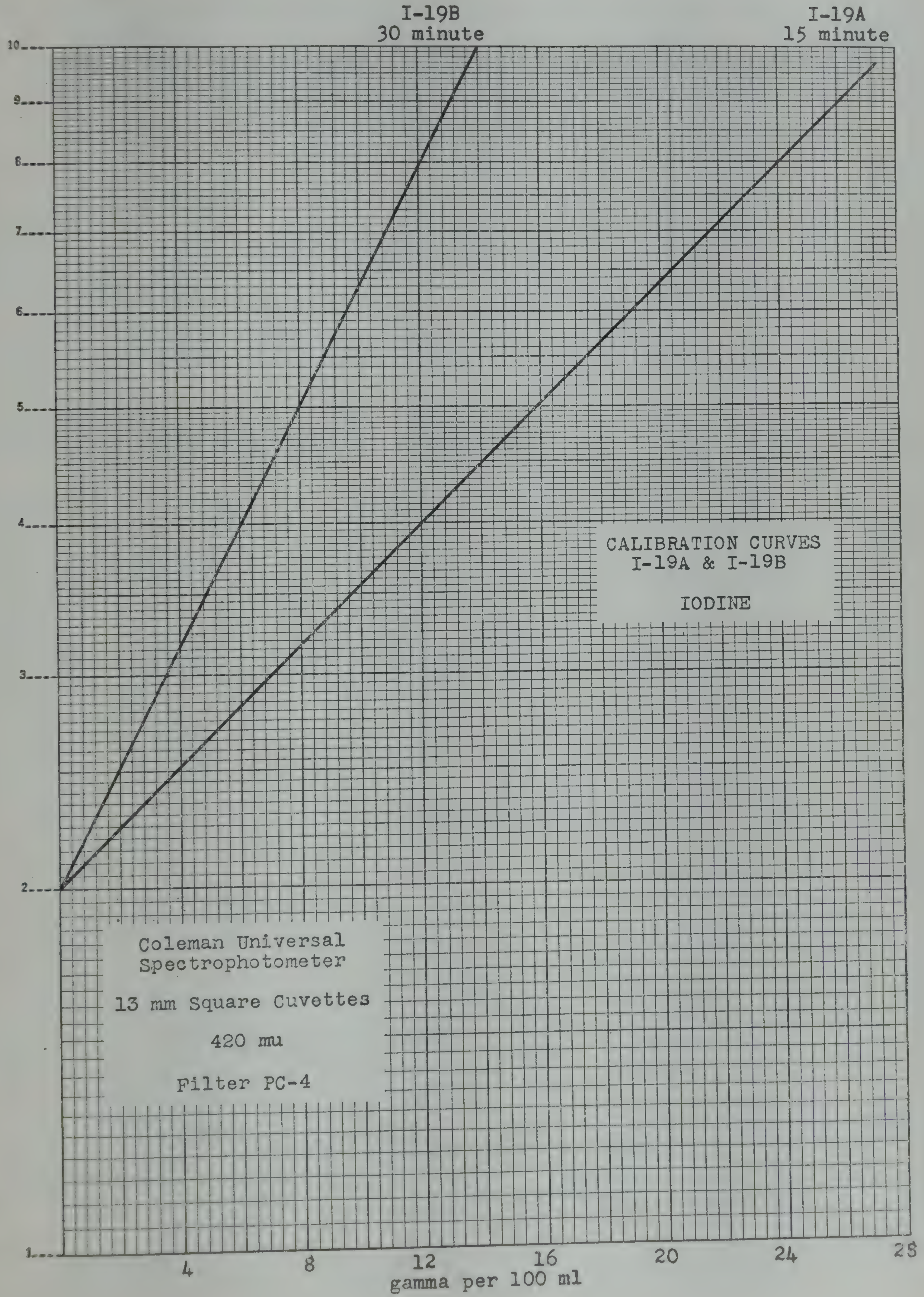
NOTES


I-19B
30 minute
I-19A
15 minute
10
9
8
7
6
5
4
3
2
1
CALIBRATION CURVES
I-19A & I-19B
IODINE
Coleman Universal
Spectrophotometer
13 mm Square Cuvettes
420 mu
Filter PC-4
4
8
12
16
20
24
28
gamma per 100 ml

Meter Reading	0	1	2	3	4	5	6	7	8	9
0										
10										
20										
30										
40										
50										
60										
70										
80										
90										

IODINE, PROTEIN-BOUND

A spectrophotometric adaptation based on the methods and modifications of Leipert (1933); Sandell and Kolthoff (1937); Stevens (1937); Chaney (1940); Salter (1940); Lein (1941); Man, Smirnow, Gildea and Peters (1942); Talbot, Butler, Saltzman and Rodriguez (1944); Taurog and Chaikoff (1946) and Barker (1948)

PRINCIPLE

Blood proteins, including the protein-bound or organic iodine fraction, are precipitated by zinc sulfate-sodium hydroxide and any remaining inorganic iodine removed by repeated washing. The protein precipitate is digested with a sulfuric-chromic acid mixture and the iodine determined as outlined in the method for total iodine (Method I-19), the same analytical principles being involved.

DETERMINATION IN WHOLE BLOOD, PLASMA OR SERUM

SAMPLE

Oxalated or heparinized whole blood or plasma, or serum; plasma or serum analyses preferable. Ordinary potassium oxalate should not be used as an anticoagulant—use only the specially recrystallized salt (Reagent #1). The protein precipitation must be carried out as soon as possible. Due to the extreme sensitivity of this determination, every possible precaution should be taken to avoid contamination. Use no iodine for sterilization purposes and have no iodine (even if in a stoppered bottle) in the immediate vicinity either while withdrawing the blood or when performing the analysis. For precise results, tincture of iodine or other volatile forms of iodine should not even be in the same room.

TEST PROCEDURE

Special Apparatus

Round-bottom centrifuge tubes, 50 ml.

Ostwald-Folin pipettes accurately calibrated "to contain" 2 ml.

Chaney-Riggs distillation apparatus (Fig. I-19). For a detailed description, see Method I-19.

Graduated test tubes—25 ml in 0.5 ml; the 25 ml graduation must be accurately calibrated.

Pipette with fine tip and small orifice, the volume between two graduation marks being exactly 0.5 ml.

Cuvettes accurately graduated at 9.5 ml.

Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.

Note: See remarks in Method I-19.

Reagents

1. **Potassium Oxalate, Iodine-Free.** Same as for total iodine.
2. **Water, Distilled, Iodine-Free.** Same as for total iodine.
3. **Sulfuric Acid, 70% by Weight.** Same as for total iodine.
4. **Chromic Acid, 60% Aqueous.** Same as for total iodine.
5. **Sodium Sulfite, 10% Aqueous.** Same as for total iodine.
6. **Phosphorous Acid, 50% Aqueous.** Same as for total iodine.
7. **Sulfuric Acid, Volumetric 1 N.** Same as for total iodine.
8. **Arsenious Acid Reagent (Barker).** Same as for total iodine.
9. **Ceric Sulfate Reagent (Barker).** Same as for total iodine.
10. **Sulfuric Acid, Volumetric 0.04 N.** Same as for total iodine.
12. **Acid Zinc Sulfate Solution (Somogyi).** Dissolve 12.5 gm crystalline zinc sulfate ($ZnSO_4$-$7H_2O$—J. T. Baker, A.C.S.) in approximately 500 ml iodine-free distilled water (2); add 125 ml 0.25 N sulfuric acid (a); mix and dilute to 1000 ml; titrate against 0.75 N sodium hydroxide (13) with phenolphthalein as follows: place exactly 50 ml of the zinc sulfate solution in a titration flask and add approximately 25 ml distilled water; add the sodium hydroxide, dropwise with constant agitation, until a pink color persists for at least 1 minute; adjust so that 50 ml of the zinc sulfate require 6.7–6.8 ml of the sodium hydroxide and re-titrate. Preserve in Pyrex glass-stoppered bottle.
    (a) Sulfuric acid, volumetric 0.25 N; dilute 250 ml 1 N sulfuric acid (7) to 1000 ml with iodine-free distilled water (2) and mix.
13. **Sodium Hydroxide, Volumetric 0.75 N (Somogyi).** Dilute 750 ml 1 N sodium hydroxide (8a) to 1000 ml with iodine-free distilled water (2) and mix. Preserve in Pyrex glass-stoppered bottle.

Technic

Note: If inorganic iodine (Method I-21) is to be determined on the same sample, reserve the combined protein-free filtrate and washings in Step 10.

For concentrations of protein-bound iodine less than 2.5 gamma per 100 ml, use a 30 minute reduction period and obtain the values directly from the calibration table for total iodine prepared from curve I-19B; for concentrations in excess of 25 gamma per 100 ml, use a 2.5 ml aliquot and 2.5 ml 0.04 N sulfuric acid (10) instead of a 5 ml aliquot and multiply the values obtained from the calibration table for total iodine prepared from curve I-19A by 2.

1. In a 50 ml round-bottom centrifuge tube (sample) place
    - 16 ml acid zinc sulfate solution (12)
    - 2 ml sample from an Ostwald-Folin pipette, thoroughly rinsing out the pipette by drawing in and expelling some of the mixture several times.
2. Mix thoroughly by gentle agitation and allow to stand for 5 minutes.
3. Add 2 ml 0.75 N sodium hydroxide (13); stopper with a clean rubber stopper and shake vigorously until foam-free.
4. In another 50 ml round-bottom centrifuge tube (reagent blank) place
    - 16 ml acid zinc sulfate solution (12)
    - 2 ml iodine-free distilled water (2)
    - 2 ml 0.75 N sodium hydroxide (13) and mix thoroughly.
5. Allow both tubes to stand for 10 minutes.
6. Centrifuge at 2000 rpm for 10 minutes.
7. Quantitatively decant the supernatant fluids into correspondingly marked 250 ml Erlenmeyer flasks.
8. To each tube add 25 ml iodine-free distilled water (2), stir up the precipitates thoroughly and centrifuge at 2000 rpm for 10 minutes.
9. Quantitatively decant the supernatant fluids into the proper Erlenmeyer flasks.
10. Repeat Steps 8 and 9 three times—test one drop of the final washing on a black spot plate with one drop acidified 0.1 N silver nitrate; there must be no precipitate or opalescence—the appearance of any turbidity indicates that further washing is necessary. RESERVE THE WASHINGS IN THE ERLENMEYER FLASKS FOR INORGANIC IODINE ANALYSIS (Method I-21).
11. After the last decantation, add 5 ml 70% sulfuric acid (3) to each tube and stir until the precipitates are completely dissolved.
12. Quantitatively transfer to correspondingly marked digestion flasks, washing out each tube with four 5 ml portions 70% sulfuric acid (3) and finally with 5 ml iodine-free distilled water (2), adding the washings to the appropriate digestion flask.

Note: From this point on, both sample and reagent blank are treated in an identical manner.

13. Proceed exactly as outlined in the test procedure for total iodine (Method I-19), Steps 3–29 inclusive.
14. Obtain the values for the recorded transmittancy readings from the calibration table for total iodine (for the 15 minute reduction period use the calibration table prepared from Calibration Curve I-19A; for the 30 minute period use the table prepared from Calibration Curve I-19B) and calculate the concentration of protein-bound iodine as follows.

Calculation

Value for sample in gamma per 100 ml (result (b)) — Value for reagent blank in gamma per 100 ml (result (a)) = Protein-bound iodine in gamma per 100 ml sample

CALIBRATION PROCEDURE

This calibration procedure need not be carried out. The values are obtained directly from the calibration tables for total iodine (Method I-19).

◆

PROCEDURE FOR USE WITH LINEAR-SCALE PHOTOELECTRIC COLORIMETERS (SHEARD-SANFORD TYPE)

TEST PROCEDURE

Exactly as outlined; set the reference blank at 20 and use a blue filter.

CALIBRATION PROCEDURE

Need not be performed; values obtained directly from the calibration tables for total iodine.

◆

PROCEDURE FOR USE WITH LOGARITHMIC-SCALE PHOTOELECTRIC COLORIMETERS (KLETT-SUMMERSON TYPE)

TEST PROCEDURE

Exactly as outlined; set the reference blank at 300 and use a blue filter.

CALIBRATION PROCEDURE

Need not be performed; values obtained directly from the calibration tables for total iodine.

BIBLIOGRAPHY

Same as listed under Total Iodine (Method I-19).

IODINE, INORGANIC

A spectrophotometric adaptation based on the methods and modifications of Leipert (1933); Sandell and Kolthoff (1937); Stevens (1937); Chaney (1940); Salter (1940); Lein (1941); Man, Smirnow, Gildea and Peters (1942); Talbot, Butler, Saltzman and Rodriguez (1944); Taurog and Chaikoff (1946) and Barker (1948)

PRINCIPLE

Blood proteins, including the protein-bound or organic iodine fraction, are precipitated by zinc sulfate-sodium hydroxide and any remaining inorganic iodine removed by repeated washing. The protein-free filtrate is digested with a sulfuric-chromic acid mixture and the iodine determined as outlined in the method for total iodine (Method I-19), the same analytical principles being involved. Note: Never determine either the inorganic or protein-bound fraction by difference; the sum of the two fractions must equal the total iodine as found by actual determination.

DETERMINATION IN WHOLE BLOOD, PLASMA OR SERUM

SAMPLE

Oxalated or heparinized whole blood or plasma, or serum. When the subject is under iodine medication, whole blood analyses should be made; otherwise plasma or serum analyses are preferable. Ordinary potassium oxalate should not be used as an anticoagulant—use only the specially recrystallized salt (Reagent #1). The protein precipitation must be carried out as soon as possible. Due to the extreme sensitivity of this determination, every possible precaution should be taken to avoid contamination. Use no iodine for sterilization purposes and have no iodine (even if in a stoppered bottle) in the immediate vicinity either while withdrawing the blood or when performing the analysis. For precise results, tincture of iodine or other volatile forms of iodine should not even be in the same room.

TEST PROCEDURE

Special Apparatus

Round-bottom centrifuge tubes, 50 ml.
Ostwald-Folin pipettes accurately calibrated "to contain" 2 ml.
Chaney-Riggs distillation apparatus (Fig. I-19). For a detailed description, see Method I-19.
Graduated test tubes—25 ml in 0.5 ml; the 25 ml graduation must be accurately calibrated.
Pipette with fine tip and small orifice, the volume between two graduation marks being exactly 0.5 ml.
Cuvettes accurately graduated at 9.5 ml.
Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.

Note: See remarks in Method I-19.

Reagents

1. **Potassium Oxalate, Iodine-Free.** Same as for protein-bound iodine.
2. **Water, Distilled, Iodine-Free.** Same as for protein-bound iodine.
3. **Sulfuric Acid, 70% by Weight.** Same as for protein-bound iodine.
4. **Chromic Acid, 60% Aqueous.** Same as for protein-bound iodine.
5. **Sodium Sulfite, 10% Aqueous.** Same as for protein-bound iodine.
6. **Phosphorous Acid, 50% Aqueous.** Same as for protein-bound iodine.
7. **Sulfuric Acid, Volumetric 1 N.** Same as for protein-bound iodine.
8. **Arsenious Acid Reagent (Barker).** Same as for protein-bound iodine.
9. **Ceric Sulfate Reagent (Barker).** Same as for protein-bound iodine.
10. **Sulfuric Acid, Volumetric 0.04 N.** Same as for protein-bound iodine.
12. **Acid Zinc Sulfate Solution (Somogyi).** Same as for protein-bound iodine.
13. **Sodium Hydroxide, Volumetric 0.75 N (Somogyi).** Same as for protein-bound iodine.

Technic

Note: If the analysis is to be carried out on the protein-free filtrate as reserved in the protein-bound iodine determination (Method 1-20, Step 10), omit the following Steps 1–10 inclusive.

For concentrations of inorganic iodine less than 2.5 gamma per 100 ml, use a 30 minute reduction period and obtain the values directly from the calibration table for total iodine prepared from curve I-19B; for concentrations in excess of 25 gamma per 100 ml, use a 2.5 ml aliquot and 2.5 ml 0.04 N sulfuric acid (10) instead of a 5 ml aliquot and multiply the values obtained from the calibration table for total iodine prepared from curve I-19A by 2.

If the inorganic iodine content is expected to be definitely high (after iodine medication), use an aliquot of the protein-free filtrate in Step 11 and correct the values obtained from the calibration tables accordingly.

1. In a 50 ml round-bottom centrifuge tube (sample) place
   16 ml acid zinc sulfate solution (12)
   2 ml sample from an Ostwald-Folin pipette, thoroughly rinsing out the pipette by drawing in and expelling some of the mixture several times.
2. Mix thoroughly by gentle agitation and allow to stand for 5 minutes.

3. Add 2 ml 0.75 N sodium hydroxide (13); stopper with a clean rubber stopper and shake vigorously until foam-free.
4. In another 50 ml round-bottom centrifuge tube (reagent blank) place
    16 ml acid zinc sulfate solution (12)
    2 ml iodine-free distilled water (2)
    2 ml 0.75 N sodium hydroxide (13) and mix thoroughly.
5. Allow both tubes to stand for 10 minutes.
6. Centrifuge at 2000 rpm for 10 minutes.
7. Quantitatively decant the supernatant fluids into correspondingly marked 250 ml Erlenmeyer flasks.
8. To each tube add 25 ml iodine-free distilled water (2), stir up the precipitates thoroughly and centrifuge at 2000 rpm for 10 minutes.
9. Quantitatively decant the supernatant fluids into the proper Erlenmeyer flasks.
10. Repeat Steps 8 and 9 three times—test one drop of the final washing on a black spot plate with one drop acidified 0.1 N silver nitrate; there must be no precipitate or opalescence—the appearance of any turbidity indicates that further washing is necessary. RESERVE THE PROTEIN PRECIPITATE FOR PROTEIN-BOUND IODINE ANALYSIS (Method I-20).
11. Evaporate the combined protein-free filtrate and washings on a hot plate at low heat to a volume of approximately 10 ml.
12. Quantitatively transfer to correspondingly marked digestion flasks, washing out each flask with four 5 ml portions 70% sulfuric acid (3) and finally with 5 ml iodine-free distilled water (2), adding the washings to the appropriate digestion flask.

Note: From this point on, both sample and reagent blank are treated in an identical manner.

13. Proceed exactly as outlined in the test procedure for total iodine (Method I-19), Steps 3–29 inclusive.
14. Obtain the values for the recorded transmittancy readings from the calibration table for total iodine (for the 15 minute reduction period use the calibration table prepared from Calibration Curve I-19A; for the 30 minute period use the table prepared from Calibration Curve I-19B) and calculate the concentration of inorganic iodine as follows.

Calculation

Value for sample in gamma per 100 ml (result (b)) — Value for reagent blank in gamma per 100 ml (result (a)) = Inorganic iodine in gamma per 100 ml sample

CALIBRATION PROCEDURE

This calibration procedure need not be carried out. The values are obtained directly from the calibration tables for total iodine (Method I-19).

PROCEDURE FOR USE WITH LINEAR-SCALE PHOTOELECTRIC COLORIMETERS (SHEARD-SANFORD TYPE)

TEST PROCEDURE

Exactly as outlined; set the reference blank at 20 and use a blue filter.

CALIBRATION PROCEDURE

Need not be performed; values obtained directly from the calibration tables for total iodine.

PROCEDURE FOR USE WITH LOGARITHMIC-SCALE PHOTOELECTRIC COLORIMETERS (KLETT-SUMMERSON TYPE)

TEST PROCEDURE

Exactly as outlined; set the reference blank at 300 and use a blue filter.

CALIBRATION PROCEDURE

Need not be performed; values obtained directly from the calibration tables for total iodine.

BIBLIOGRAPHY

Same as listed under Total Iodine (Method I-19).

IRON, NON-HEMOGLOBIN

A spectrophotometric adaptation based on the methods and modifications of Barkan (1927; 1937), Hill (1931), Borgen and Elvehjem (1937), Jackson (1938), Kohler, Elvehjem and Hart (1939), Koenig and Johnson (1942) and Moss and Mellon (1942)

PRINCIPLE

Plasma or serum is incubated with dilute hydrochloric acid and after precipitation of the protein, the ferric iron is reduced to the ferrous form by means of hydroquinone. The ferrous iron is then treated with a-a′-dipyridyl to form an intense red complex (Blau reaction).

DETERMINATION IN PLASMA OR SERUM

SAMPLE

Plasma or serum from blood withdrawn during post-absorptive state. The following precautions should be carefully observed to prevent hemolysis; slight traces are of no consequence but samples showing gross hemolysis cannot be used. Withdraw blood with minimum stasis, using absolutely dry syringes and stainless steel needles (gold or platinum needles are preferable) and allow the syringe to fill without exerting suction with the plunger. Remove needle from syringe and allow blood to flow slowly down the side of the collection tube without pressure on the plunger. For plasma analyses use sodium citrate as anticoagulant—fluorides, oxalates and tungstates interfere; analyses preferably made on serum. The plasma or serum must be separated from the red cells within one hour after collection of the sample.

TEST PROCEDURE

Special Apparatus

Ostwald-Folin pipettes accurately calibrated "to contain" 4 ml.

Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.

Note: Glassware should be thoroughly cleaned with 25% hydrochloric acid, rinsed at least three times with iron-free distilled water (Reagent #1) and dried before use.

Reagents

1. **Water, Distilled, Iron-Free.** Redistil distilled water in an all-glass Pyrex distillation apparatus until iron-free. To test for the presence of iron, treat 10 ml with 1 ml concentrated hydrochloric acid, 0.1 ml concentrated nitric acid, 4 ml 3 N potassium thiocyanate and 2 ml iso-amyl alcohol; shake thoroughly and allow the alcohol layer to separate; a colorless alcohol layer indicates the absence of iron. Use for the preparation of all reagents and standards and where indicated in the test procedure.
2. **Hydrochloric Acid, Volumetric 0.3 N.** Slowly add 30 ml concentrated hydrochloric acid (HCl—Baker & Adamson, A.C.S.) to 1000 ml iron-free distilled water (1) and mix; titrate against standard 0.1 N alkali using phenolphthalein as indicator; adjust accordingly and re-titrate—10 ml of 0.3 N acid require 30 ml of 0.1 N alkali.
3. **Trichloracetic Acid, 20% Aqueous.** Dilute 20 ml concentrated trichloracetic acid (a) to 100 ml with iron-free distilled water (1). Preserve in glass-stoppered bottle. Prepare fresh monthly.
   (a) Trichloracetic acid, concentrated; dissolve 100 gm crystalline trichloracetic acid (CCl_3COOH—J. T. Baker, CP Analyzed) in iron-free distilled water (1) and dilute to 100 ml. Preserve in glass-stoppered bottle. Stability indefinite.
4. **Sodium Acetate, Saturated Aqueous.** In an 8 ounce bottle place 130 gm finely pulverized crystalline sodium acetate ($NaC_2H_3O_2 \cdot 3H_2O$—Baker & Adamson, A.C.S.) and add 100 ml iron-free distilled water (1) previously heated to 45° C.; shake thoroughly and allow to cool and settle. Use the clear supernatant fluid. Note: Complete saturation is essential.
5. **Hydroquinone, 1% Aqueous.** In a 50 ml mixing cylinder place 0.5 gm white hydroquinone crystals (C_6H_4-1,4-$(OH)_2$—Eastman #356); add approximately 45 ml iron-free distilled water (1); shake until dissolved and dilute to volume. A slight opacity which sometimes appears may be disregarded. This solution must be prepared fresh daily, preferably just before use.
6. **a-a′-Dipyridyl, 0.2% Aqueous.** Dissolve 200 mg a-a′-dipyridyl ($(C_5H_4N)_2$—G. Frederick Smith, Reagent) in 10 ml iron-free distilled water (1) with the aid of heat, if necessary; quantitatively transfer to a 100 ml volumetric flask; dilute to volume with iron-free distilled water and mix. Discard if any trace of color is apparent. Preserve in a brown glass bottle in refrigerator.

Technic

Note: For concentrations of iron in excess of 1 mg per 100 ml, use 2 ml centrifugate, 0.5 ml 20% trichloracetic acid (3) and 1.5 ml iron-free distilled water (1) in Step 7 and multiply the values obtained from the calibration table by 2.

1. In a 15 ml centrifuge tube place
   2 ml 0.3 N hydrochloric acid (2)
   4 ml sample from an Ostwald-Folin pipette, thoroughly rinsing out the pipette by drawing in and expelling some of the mixture several times; mix thoroughly.

2. Allow to stand in the incubator at 37° C. for 1 hour.
3. Add 2 ml 20% trichloracetic acid (3), drop by drop while constantly agitating the tube.
4. Stopper the tube with a clean cork covered with aluminum foil and shake vigorously.
5. Allow to stand at room temperature for 1 hour and centrifuge the foil-stoppered tube at 3000 rpm for 15 minutes—filtration may be employed if iron-free paper is used.
6. In a cuvette (sample) place
   4 ml clear centrifugate.
7. In another cuvette (reagent blank) place
   2 ml iron-free distilled water (1)
   1 ml 0.3 N hydrochloric acid (2)
   1 ml 20% trichloracetic acid (3) and mix.
8. To each cuvette add in the order named, mixing after each addition
   0.5 ml saturated sodium acetate (4)
   0.3 ml 1% hydroquinone (5)
   1 ml 0.2% a-a′-dipyridyl (6).
9. Allow to stand at room temperature for 1 hour.
10. Measure the transmittancy of the sample against the reagent blank set at 100% transmission at a wave length of **520 mμ** and obtain the concentration of non-hemoglobin iron from the calibration table.

CALIBRATION PROCEDURE

Special Apparatus

Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.

Reagents

1. **Water, Distilled, Iron-Free.** Same as for Test Procedure.
2. **Hydrochloric Acid, Volumetric 0.3 N.** Same as for Test Procedure.
3. **Trichloracetic Acid, 20% Aqueous.** Same as for Test Procedure.
4. **Sodium Acetate, Saturated Aqueous.** Same as for Test Procedure.
5. **Hydroquinone, 1% Aqueous.** Same as for Test Procedure.
6. **a-a′-Dipyridyl, 0.2% Aqueous.** Same as for Test Procedure.
7. **Iron Standards**
   (a) Iron stock standard, Dupray (1 ml contains 1 mg ferric iron). Thoroughly clean a small piece of iron wire of known iron content (special for Standardization, usually 99.85% Fe—Mallinckrodt, AR) with fine emery cloth or paper until shining and free of rust and dirt. Knowing the iron content, weigh out an amount of wire that contains 100 mg of iron. In a 250 ml Florence flask place 10 ml concentrated iron-free nitric acid (HNO_3—Grasselli, A.C.S.) and approximately 40 ml iron-free distilled water (1); heat to boiling; roll the wire into a loose coil and drop, together with any fine trimmings, into the boiling acid; boil until completely dissolved and then one minute longer. Cool and transfer quantitatively to a 100 ml volumetric flask; dilute to volume with iron-free distilled water and mix. Stable indefinitely.
   (b) Dilute standard (1 ml contains 0.01 mg ferric iron). In a 100 ml volumetric flask place 1 ml stock standard (7a) and dilute to volume with iron-free distilled water (1). Prepare just before use.
   (c) Calibration standards. In a series of seven accurately calibrated 10 ml volumetric flasks place respectively 0, 1, 2, 4, 6, 8 and 10 ml of the dilute standard (7b) and dilute to volume with iron-free distilled water (1). These standards represent concentrations of iron equivalent to 0, 0.1, 0.2, 0.4, 0.6, 0.8 and 1.0 mg per 100 ml sample. Prepare just before use.

Technic

1. Transfer 2 ml of each calibration standard (7c) to a correspondingly marked cuvette.
2. To each cuvette add in the order named, mixing after each addition
   1 ml 0.3 N hydrochloric acid (2)
   1 ml 20% trichloracetic acid (3)
   0.5 ml saturated sodium acetate (4)
   0.3 ml 1% hydroquinone (5)
   1 ml 0.2% a-a′-dipyridyl (6).
3. Allow to stand at room temperature for 1 hour.
4. Set the zero concentration standard at 100% transmission at a wave length of **520 mμ** and record the transmittancy of the 0.1, 0.2, 0.4, 0.6, 0.8 and 1.0 mg per cent standards. The transmittancy of the 1.0 mg standard should be approximately 26%.
5. Plot the observed values on semi-log graph paper—the points fall on a straight line which passes through the origin (Calibration Curve I-25).
6. Prepare a calibration table from the T-C values obtained from the plotted curve.

PROCEDURE FOR USE WITH LINEAR-SCALE PHOTOELECTRIC COLORIMETERS (SHEARD-SANFORD TYPE)

TEST PROCEDURE

Exactly as outlined; set the reagent blank at 100 and use a green filter.

CALIBRATION PROCEDURE

Exactly as outlined—use a green filter; plot the observed readings on semi-log graph paper—the points fall on a straight line passing through the origin.

PROCEDURE FOR USE WITH LOGARITHMIC-SCALE PHOTOELECTRIC COLORIMETERS (KLETT-SUMMERSON TYPE)

TEST PROCEDURE

Exactly as outlined; set the reagent blank at 0 and use a green filter.

CALIBRATION PROCEDURE

Exactly as outlined—use a green filter; plot the observed readings on linear graph paper—the points fall on a straight line passing through the origin.

Note: For instruments with cells, cuvettes or tubes requiring a final volume of more than 5.8 ml, follow the procedure as outlined and add 2 ml iron-free distilled water just before measurements are made in both the test and calibration procedures.

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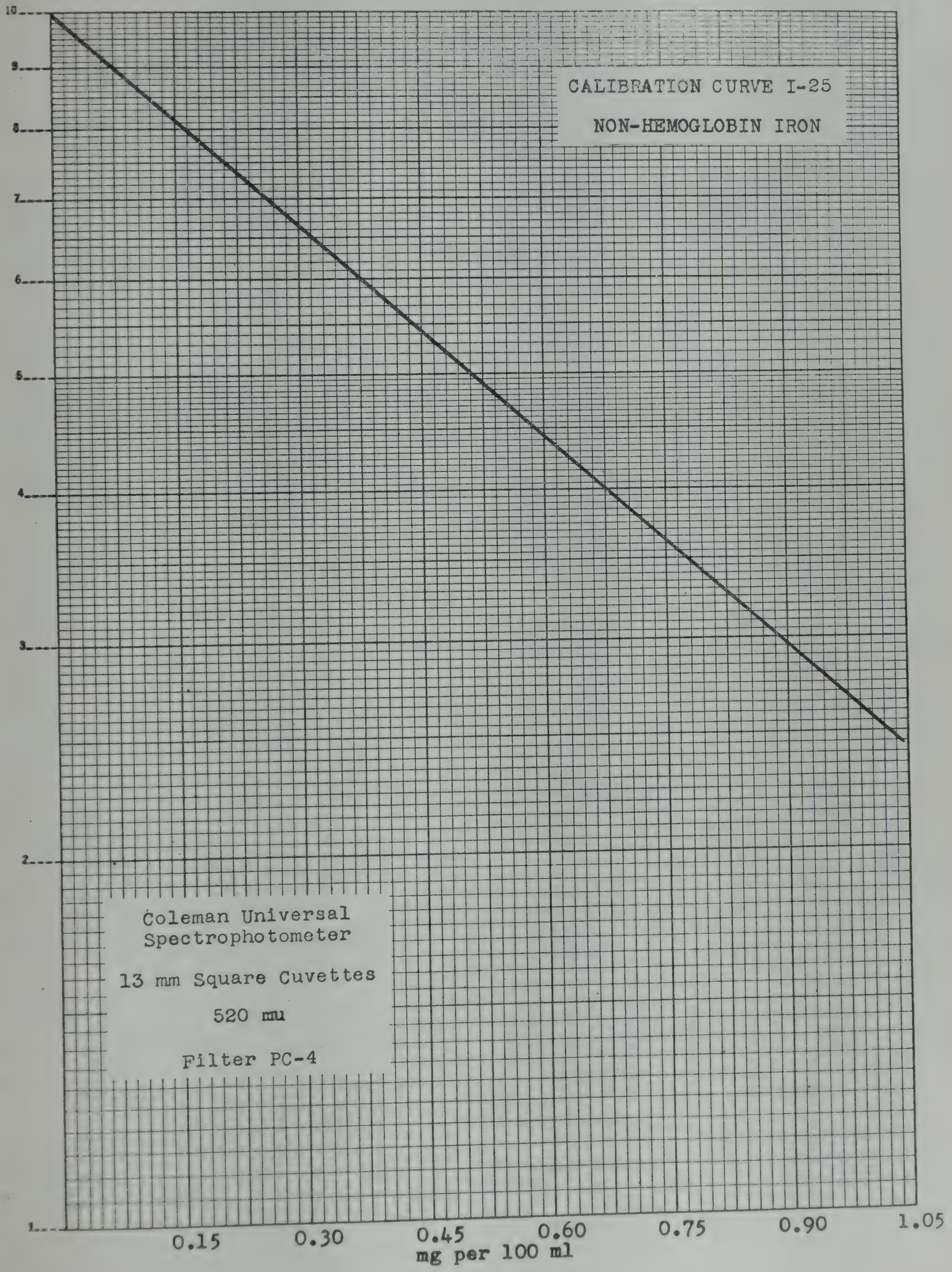
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NON-HEMOGLOBIN IRON
Coleman Universal Spectrophotometer
13 mm Square Cuvettes
520 mu
Filter PC-4
10
9
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6
5
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2
1
0.15
0.30
0.45
0.60
0.75
0.90
1.05
mg per 100 ml

Meter Reading	0	1	2	3	4	5	6	7	8	9
0										
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20										
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40										
50										
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70										
80										
90										

IRON, NON-HEMOGLOBIN

A spectrophotometric adaptation based on the methods and modifications of Barkan (1927; 1937), Walker (1938), Hummel and Willard (1938), Fortune and Mellon (1938), Barkan and Walker (1939; 1940), Schales (1940), Sachs, Levine, Andersen and Schmit (1941) and Moss and Mellon (1942)

PRINCIPLE

Plasma or serum is incubated with dilute hydrochloric acid and after precipitation of the protein, the ferric iron is reduced to the ferrous form by means of ascorbic acid. The ferrous iron is then treated with o-phenanthroline to form an orange-red complex (Blau reaction).

DETERMINATION IN PLASMA OR SERUM

SAMPLE

Plasma or serum from blood withdrawn during post-absorptive state. The following precautions should be carefully observed to prevent hemolysis; slight traces are of no consequence but samples showing gross hemolysis cannot be used. Withdraw blood with minimum stasis, using absolutely dry syringes and stainless steel needles (gold or platinum needles are preferable) and allow the syringe to fill without exerting suction with the plunger. Remove needle from syringe and allow blood to flow slowly down the side of the collection tube without pressure on the plunger. For plasma analyses use sodium citrate as anticoagulant—fluorides, oxalates and tungstates interfere; analyses preferably made on serum. The plasma or serum must be separated from the red cells within one hour after collection of the sample.

TEST PROCEDURE

Special Apparatus

Ostwald-Folin pipettes accurately calibrated "to contain" 4 ml.

Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.

Note: Glassware should be thoroughly cleaned with 25% hydrochloric acid, rinsed at least three times with iron-free distilled water (Reagent #1) and dried before use.

Reagents

1. **Water, Distilled, Iron-Free.** Redistil distilled water in an all-glass Pyrex distillation apparatus until iron-free. To test for the presence of iron, treat 10 ml with 1 ml concentrated hydrochloric acid, 0.1 ml concentrated nitric acid, 4 ml 3 N potassium thiocyanate and 2 ml iso-amyl alcohol; shake thoroughly and allow the alcohol layer to separate; a colorless alcohol layer indicates the absence of iron. Use for the preparation of all reagents and standards and where indicated in the test procedure.
2. **Hydrochloric Acid, Volumetric 0.3 N.** Slowly add 30 ml concentrated hydrochloric acid (HCl—Baker & Adamson, A.C.S.) to 1000 ml iron-free distilled water (1) and mix; titrate against standard 0.1 N alkali using phenolphthalein as indicator; adjust accordingly and re-titrate—10 ml of 0.3 N acid require 30 ml of 0.1 N alkali.
3. **Trichloracetic Acid, 20% Aqueous.** Dilute 20 ml concentrated trichloracetic acid (a) to 100 ml with iron-free distilled water (1). Preserve in glass-stoppered bottle. Prepare fresh monthly.
    (a) Trichloracetic acid, concentrated; dissolve 100 gm crystalline trichloracetic acid (CCl_3-$COOH$—J. T. Baker, CP Analyzed) in iron-free distilled water (1) and dilute to 100 ml. Preserve in glass-stoppered bottle. Stability indefinite.
4. **Sodium Acetate, Saturated Aqueous.** In an 8 ounce bottle place 130 gm finely pulverized crystalline sodium acetate ($NaC_2H_3O_2 \cdot 3H_2O$—Baker & Adamson, A.C.S.) and add 100 ml iron-free distilled water (1) previously heated to 45° C.; shake thoroughly and allow to cool and settle. Use the clear supernatant fluid. Note: Complete saturation is essential.
5. **Ascorbic Acid, 1% Aqueous.** Dissolve 100 mg crystalline ascorbic acid ($C_6H_8O_6$—Merck, U.S.P.) in 10 ml iron-free distilled water (1). Prepare fresh daily.
6. **o-Phenanthroline, 0.1% Aqueous.** Dissolve 100 mg 1,10-phenanthroline ($C_{12}H_8N_2 \cdot H_2O$—G. Frederick Smith, Reagent) in 10 ml iron-free distilled water (1) with the aid of heat, if necessary; quantitatively transfer to a 100 ml volumetric flask; dilute to volume with iron-free distilled water and mix. Discard if any trace of color is apparent. Preserve in a brown glass bottle in refrigerator.

Technic

Note: For concentrations of iron in excess of 1 mg per 100 ml, use 2 ml centrifugate, 0.5 ml 20% trichloracetic acid (3) and 1.5 ml iron-free distilled water (1) in Step 7 and multiply the values obtained from the calibration table by 2.

1. In a 15 ml centrifuge tube place
    2 ml 0.3 N hydrochloric acid (2)
    4 ml sample from an Ostwald-Folin pipette, thoroughly rinsing out the pipette by drawing in and expelling some of the mixture several times; mix thoroughly.
2. Allow to stand in the incubator at 37° C. for 1 hour.

3. Add 2 ml 20% trichloracetic acid (3), drop by drop while constantly agitating the tube.
4. Stopper the tube with a clean cork covered with aluminum foil and shake vigorously.
5. Allow to stand at room temperature for 1 hour and centrifuge the foil-stoppered tube at 3000 rpm for 15 minutes—filtration may be employed if iron-free paper is used.
6. In a cuvette (sample) place
   4 ml clear centrifugate.
7. In another cuvette (reagent blank) place
   2 ml iron-free distilled water (1)
   1 ml 0.3 N hydrochloric acid (2)
   1 ml 20% trichloracetic acid (3) and mix.
8. To each cuvette add in the order named, mixing after each addition
   0.5 ml saturated sodium acetate (4)
   0.3 ml 1% ascorbic acid (5)
   1 ml 0.1% o-phenanthroline (6).
9. Allow to stand at room temperature for 1 hour.
10. Measure the transmittancy of the sample against the reagent blank set at 100% transmission at a wave length of **510 mμ** and obtain the concentration of non-hemoglobin iron from the calibration table.

CALIBRATION PROCEDURE

Special Apparatus

Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.

Reagents

1. **Water, Distilled, Iron-Free.** Same as for Test Procedure.
2. **Hydrochloric Acid, Volumetric 0.3 N.** Same as for Test Procedure.
3. **Trichloracetic Acid, 20% Aqueous.** Same as for Test Procedure.
4. **Sodium Acetate, Saturated Aqueous.** Same as for Test Procedure.
5. **Ascorbic Acid, 1% Aqueous.** Same as for Test Procedure.
6. **o-Phenanthroline, 0.1% Aqueous.** Same as for Test Procedure.
7. **Iron Standards**
   (a) Iron stock standard, Dupray (1 ml contains 1 mg ferric iron). Thoroughly clean a small piece of iron wire of known iron content (Special for Standardization, usually 99.85% Fe—Mallinckrodt, AR) with fine emery cloth or paper until shining and free of rust and dirt. Knowing the iron content, weigh out an amount of wire that contains 100 mg of iron. In a 250 ml Florence flask place 10 ml concentrated iron-free nitric acid (HNO_3—Grasselli, A.C.S.) and approximately 40 ml iron-free distilled water (1); heat to boiling; roll the wire into a loose coil and drop, together with any fine trimmings, into the boiling acid; boil until completely dissolved and then one minute longer. Cool and transfer quantitatively to a 100 ml volumetric flask; dilute to volume with iron-free distilled water and mix. Stable indefinitely.
   (b) Dilute standard (1 ml contains 0.01 mg ferric iron). In a 100 ml volumetric flask place 1 ml stock standard (7a) and dilute to volume with iron-free distilled water (1). Prepare just before use.
   (c) Calibration standards. In a series of seven accurately calibrated 10 ml volumetric flasks place respectively 0, 1, 2, 4, 6, 8 and 10 ml of the diluted standard (7b) and dilute to volume with iron-free distilled water (1). These standards represent concentrations of iron equivalent to 0, 0.1, 0.2, 0.4, 0.6, 0.8 and 1.0 mg per 100 ml sample. Prepare just before use.

Technic

1. Transfer 2 ml of each calibration standard (7c) to a correspondingly marked cuvette.
2. To each cuvette add in the order named, mixing after each addition
   1 ml 0.3 N hydrochloric acid (2)
   1 ml 20% trichloracetic acid (3)
   0.5 ml saturated sodium acetate (4)
   0.3 ml 1% ascorbic acid (5)
   1 ml 0.1% o-phenanthroline (6).
3. Allow to stand at room temperature for 1 hour.
4. Set the zero concentration standard at 100% transmission at a wave length of **510 mμ** and record the transmittancy of the 0.1, 0.2, 0.4, 0.6, 0.8 and 1.0 mg per cent standards. The transmittancy of the 1.0 mg standard should be approximately 21%.
5. Plot the observed values on semi-log graph paper—the points fall on a straight line which passes through the origin (Calibration Curve I-26).
6. Prepare a calibration table from the T-C values obtained from the plotted curve.

PROCEDURE FOR USE WITH LINEAR-SCALE PHOTOELECTRIC COLORIMETERS (SHEARD-SANFORD TYPE)

TEST PROCEDURE

Exactly as outlined; set the reagent blank at 100 and use a green filter.

CALIBRATION PROCEDURE

Exactly as outlined—use a green filter; plot the observed readings on semi-log graph paper—the points fall on a straight line passing through the origin.

PROCEDURE FOR USE WITH LOGARITHMIC-SCALE PHOTOELECTRIC COLORIMETERS (KLETT-SUMMERSON TYPE)

TEST PROCEDURE

Exactly as outlined; set the reagent blank at 0 and use a green filter.

CALIBRATION PROCEDURE

Exactly as outlined—use a green filter; plot the observed readings on linear graph paper—the points fall on a straight line passing through the origin.

Note: For instruments with cells, cuvettes or tubes requiring a final volume of more than 5.8 ml, follow the procedure as outlined and add 2 ml iron-free distilled water just before measurements are made in both the test and calibration procedures.

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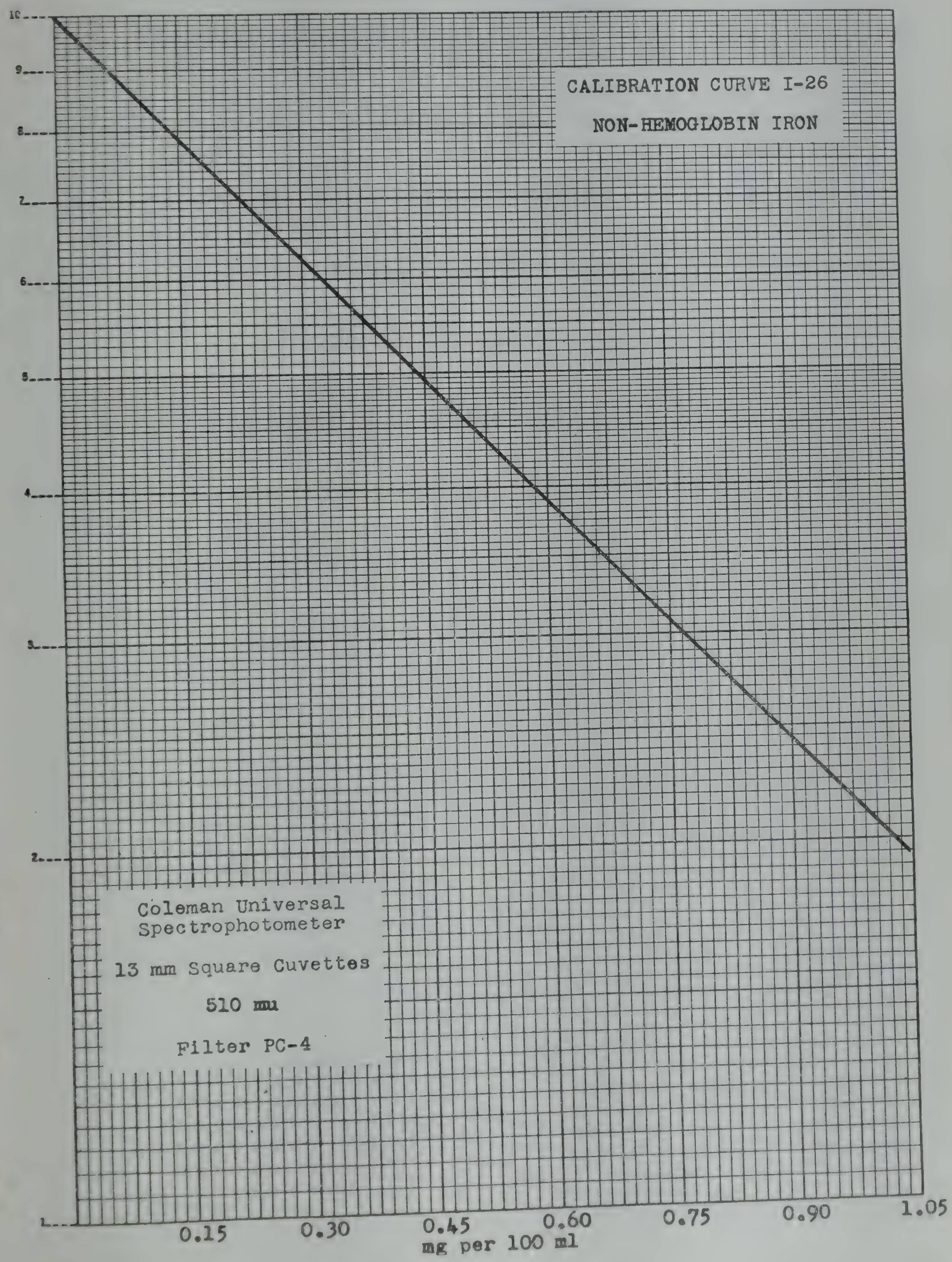
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NON-HEMOGLOBIN IRON
Coleman Universal Spectrophotometer
13 mm Square Cuvettes
510 mμ
Filter PC-4
10
9
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IRON, TOTAL

A spectrophotometric adaptation based on the methods and modifications of Wong (1923; 1928), Dupray (1927) and Hanzal (1933)

PRINCIPLE

Iron is split from protein combination by means of sulfuric acid and converted to the ferric state by potassium persulfate which also aids in the decomposition. The proteins are precipitated and the iron in the filtrate is determined by the production, in alkaline solution, of a purplish red color with thioglycolic acid (Andreasch reaction).

DETERMINATION IN WHOLE BLOOD

SAMPLE

Blood withdrawn during post-absorptive state. Anticoagulant—potassium oxalate. Platinum or gold needles are preferable to steel needles.

TEST PROCEDURE

Special Apparatus

Ostwald-Van Slyke pipettes, accurately calibrated "to deliver" 0.5 ml.
Graduated glass-stoppered cylinders; 10 ml.
Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.

Note: Glassware must be thoroughly cleaned with a mixture of hydrochloric acid and thiocyanate and rinsed at least three times with iron-free distilled water (Reagent #1).

Reagents

1. **Water, Distilled, Iron-Free.** Redistil distilled water in an all-glass Pyrex distillation apparatus until iron-free. To test for the presence of iron, treat 10 ml with 1 ml concentrated hydrochloric acid, 0.1 ml concentrated nitric acid, 4 ml 3 N potassium thiocyanate and 2 ml iso-amyl alcohol; shake thoroughly and allow the alcohol layer to separate; a colorless alcohol layer indicates the absence of iron. Use for the preparation of all reagents and standards and where indicated in the test procedure.
2. **Sulfuric Acid, Concentrated, Iron-Free** (H_2SO_4—Grasselli, A.C.S.).
3. **Potassium Persulfate, Saturated Aqueous.** Place approximately 8 gm potassium persulfate ($K_2S_2O_8$—Merck, Low Nitrogen Special, According to Van Slyke) in a brown glass bottle; add 100 ml iron-free distilled water (1) and shake thoroughly. A portion of the salt remains undissolved and settles to the bottom—do not remove the undissolved persulfate; an excess must always be present to compensate for decomposition. Shake shortly before use; allow to settle and use the clear supernatant fluid. Preserve in refrigerator.
4. **Sodium Tungstate, 10% Aqueous.** Dissolve 10 gm sodium tungstate ($Na_2WO_4 \cdot 2H_2O$—Mallinckrodt, Folin Special) in iron-free distilled water (1) and dilute to 100 ml. This solution must be neutral or slightly alkaline to phenolphthalein, not more than 0.4 ml being required to neutralize 10 ml of 0.1 N hydrochloric acid. Preserve in resistant-glass bottle.
5. **Thioglycolic Acid** ($CH_2SHCOOH$—Reagent Chemicals, Special).
6. **Ammonium Hydroxide, Concentrated, Iron-Free** (NH_4OH—Baker & Adamson, A.C.S.).

Technic

1. In a 50 ml volumetric flask (sample) place
   0.5 ml oxalated whole blood delivered from an Ostwald-Van Slyke pipette
   2 ml iron-free distilled water (1).
2. Rapidly swirl the flask until the blood is completely hemolyzed (about 2 minutes).
3. Add 2 ml concentrated sulfuric acid (2), drop by drop, while rapidly rotating the flask.
4. Agitate the flask vigorously for 5 minutes and allow to stand for 60 minutes.
5. Add 2 ml saturated potassium persulfate (3), again agitate the flask for 2 minutes and allow to stand for 30 minutes.
6. Add 25 ml iron-free distilled water (1) and 2 ml 10% sodium tungstate (4), while rapidly rotating the flask.
7. Allow to stand for 30 minutes; cool to room temperature under running tap water if necessary.
8. Carefully, so as to prevent foaming, dilute to 50 ml with iron-free distilled water (1) and mix thoroughly by repeated inversion.
9. In another 50 ml volumetric flask (reagent blank) place
   25 ml iron-free distilled water (1)
   2 ml concentrated sulfuric acid (2)
   2 ml saturated potassium persulfate (3), mix, cool and dilute to 50 ml with iron-free distilled water.
10. Filter the contents of each flask through identical acid-washed Whatman #40 papers into correspondingly marked dry 125 ml Erlenmeyer flasks, refiltering the first few drops if necessary.
11. Transfer 5 ml of each filtrate to a correspondingly marked 10 ml glass-stoppered cylinder.
12. To each cylinder add
    0.1 ml thioglycolic acid (5)
    2 ml concentrated ammonium hydroxide (6) and mix by lateral shaking.

13. Cool to room temperature under running tap water.
14. Within the next hour measure the transmittancy of the sample, after shaking thoroughly, against the reagent blank, which should be colorless, set at 100% transmission at a wave length of **535 mμ** and obtain the concentration of iron from the calibration table.

Note: The color may fade slightly on standing but is readily restored by shaking in air.

CALIBRATION PROCEDURE

Special Apparatus

Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.
Graduated glass-stoppered cylinders; 10 ml.

Reagents

1. **Water, Distilled, Iron-Free.** Same as for Test Procedure.
2. **Sulfuric Acid, Concentrated, Iron-Free.** Same as for Test Procedure.
3. **Potassium Persulfate, Saturated Aqueous.** Same as for Test Procedure.
5. **Thioglycolic Acid.** Same as for Test Procedure.
6. **Ammonium Hydroxide, Concentrated, Iron-Free.** Same as for Test Procedure.
7. **Nitric Acid, Concentrated, Iron-Free** (HNO_3—Grasselli, A.C.S.).
8. **Iron Standards**
    (a) Iron stock standard, Dupray (1 ml contains 1 mg ferric iron). Thoroughly clean a small piece of iron wire of known iron content (Special for Standardization, usually 99.85% Fe—Mallinckrodt, AR) with fine emery cloth or paper until shining and free of rust and dirt. Knowing the iron content, weigh out an amount of wire that contains 100 mg of iron. In a 250 ml Florence flask place 10 ml concentrated iron-free nitric acid (7) and approximately 40 ml iron-free distilled water (1); heat to boiling; roll the wire into a loose coil and drop, together with any fine trimmings, into the boiling acid; boil until completely dissolved and then one minute longer. Cool and transfer quantitatvely to a 100 ml volumetric flask; dilute to volume with iron-free distilled water and mix. Stable indefinitely.
    (b) Dilute standard (1 ml contains 0.1 mg ferric iron). In a 100 ml volumetric flask place 10 ml stock standard (8a) and dilute to volume with iron-free distilled water (1). Prepare day of use.
    (c) Calibration standards. In a series of seven accurately calibrated 50 ml volumetric flasks place respectively 0, 1, 1.5, 2, 2.5, 3 and 4 ml of the dilute standard (8b); add 2 ml concentrated sulfuric acid (2) and 2 ml saturated potassium persulfate (3); dilute to volume with iron-free distilled water (1). These standards represent concentrations of iron equivalent to 0, 20, 30, 40, 50, 60 and 80 mg per 100 ml sample. Prepare just before use.

Technic

1. Transfer 5 ml of each calibration standard (8c) to a correspondingly marked 10 ml glass-stoppered cylinder.
2. Proceed exactly as outlined in the Test Procedure, Steps 12–13 inclusive.
3. Set the zero concentration standard at 100% transmission at a wave length of **535 mμ** and record the transmittancy of the 20, 30, 40, 50, 60 and 80 mg per cent standards, after shaking thoroughly. The transmittancy of the 80 mg standard should be approximately 35%.
4. Plot the observed values on semi-log graph paper—the points fall on a straight line which passes through the origin (Calibration Curve I-27).
5. Prepare a calibration table from the T-C values obtained from the plotted curve.

CONVERSION FACTORS

To convert iron in mg per 100 ml to hemoglobin in gm per 100 ml divide by 3.35 (Butterfield).
To convert iron in mg per 100 ml to oxygen capacity in volumes per cent multiply by 0.4 (Van Slyke).

PROCEDURE FOR USE WITH LINEAR-SCALE PHOTOELECTRIC COLORIMETERS (SHEARD-SANFORD TYPE)

TEST PROCEDURE

Exactly as outlined; set the reagent blank at 100 and use a green filter.

CALIBRATION PROCEDURE

Exactly as outlined—use a green filter; plot the observed readings on semi-log graph paper—the points fall on a straight line passing through the origin.

PROCEDURE FOR USE WITH LOGARITHMIC-SCALE PHOTOELECTRIC COLORIMETERS (KLETT-SUMMERSON TYPE)

TEST PROCEDURE

Exactly as outlined; set the reagent blank at 0 and use a green filter.

CALIBRATION PROCEDURE

Exactly as outlined—use a green filter; plot the observed readings on linear graph paper—the points fall on a straight line passing through the origin.

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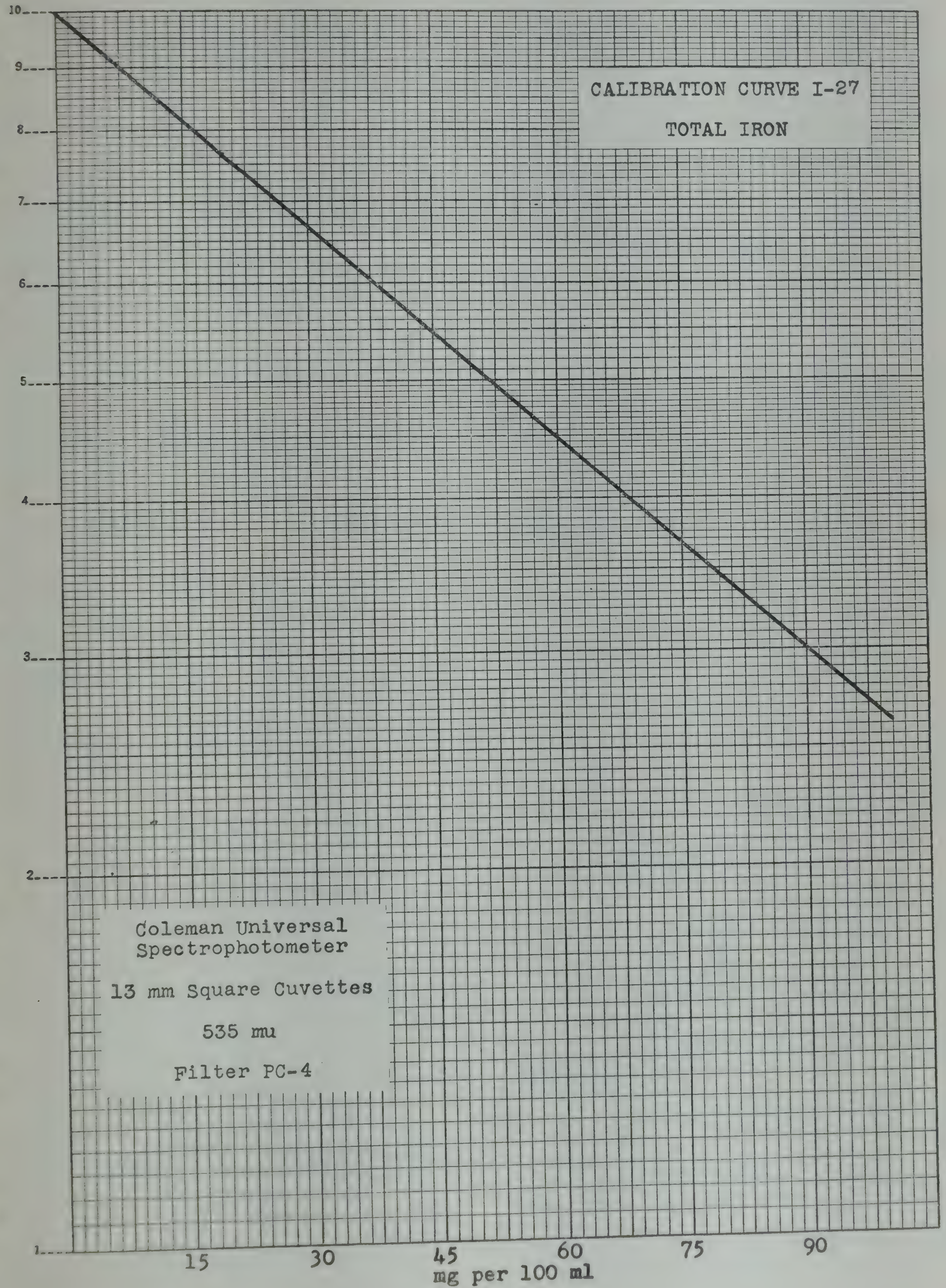
CALIBRATION CURVE I-27
TOTAL IRON
Coleman Universal Spectrophotometer
13 mm Square Cuvettes
535 mu
Filter PC-4
10
9
8
7
6
5
4
3
2
1
15
30
45
60
75
90
mg per 100 ml

Meter Reading	0	1	2	3	4	5	6	7	8	9
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IRON, TOTAL

A spectrophotometric adaptation based on the methods and modifications of Wong (1923; 1928), Dupray (1927), Walker and Fitz (1940) and Ponder (1942)

PRINCIPLE

Iron is split from protein combination by means of sulfuric acid and converted to the ferric state by potassium persulfate which also aids in the decomposition and acts as a color stabilizer. The proteins are precipitated and the iron in the filtrate is determined by the production of an orange red color with potassium thiocyanate (Ossian reaction). Acetone is added to the latter as a color intensifier and preservative.

DETERMINATION IN WHOLE BLOOD

SAMPLE

Blood withdrawn during post-absorptive state. Anticoagulant—potassium oxalate. Platinum or gold needles are preferable to steel needles.

TEST PROCEDURE

Special Apparatus

Ostwald-Van Slyke pipettes, accurately calibrated "to deliver" 0.5 ml.
Graduated glass-stoppered cylinders—25 ml; the 20 ml graduation must be accurately calibrated.
Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.

Note: Glassware must be thoroughly cleaned with a mixture of hydrochloric acid and thiocyanate and rinsed at least three times with iron-free distilled water (Reagent #1).

Reagents

1. **Water, Distilled, Iron-Free.** Redistil distilled water in an all-glass Pyrex distillation apparatus until iron-free. To test for the presence of iron, treat 10 ml with 1 ml concentrated hydrochloric acid, 0.1 ml concentrated nitric acid, 4 ml 3 N potassium thiocyanate (Reagent #5) and 2 ml iso-amyl alcohol; shake thoroughly and allow the alcohol layer to separate; a colorless alcohol layer indicates the absence of iron. Use for the preparation of all reagents and standards and where indicated in the test procedure.
2. **Sulfuric Acid, Concentrated, Iron-Free** (H_2SO_4—Grasselli, A.C.S.).
3. **Potassium Persulfate, Saturated Aqueous.** Place approximately 8 gm potassium persulfate ($K_2S_2O_8$—Merck, Low Nitrogen Special, According to Van Slyke) in a brown glass bottle; add 100 ml iron-free distilled water (1) and shake thoroughly. A portion of the salt remains undissolved and settles to the bottom—do not remove the undissolved persulfate; an excess must always be present to compensate for decomposition. Shake shortly before use; allow to settle and use the clear supernatant fluid. Preserve in refrigerator.
4. **Sodium Tungstate, 10% Aqueous.** Dissolve 10 gm sodium tungstate ($Na_2WO_4 \cdot 2H_2O$—Mallinckrodt, Folin Special) in iron-free distilled water (1) and dilute to 100 ml. This solution must be neutral or slightly alkaline to phenolphthalein, not more than 0.4 ml being required to neutralize 10 ml of 0.1 N hydrochloric acid. Preserve in resistant-glass bottle.
5. **Potassium Thiocyanate, Volumetric 3 N.** Dissolve 300 gm dry potassium thiocyanate (KCNS—Baker & Adamson, Reagent) in iron-free distilled water (1); add 40 ml acetone ($(CH_3)_2CO$—Baker & Adamson, A.C.S.); dilute to 1000 ml with iron-free distilled water. Allow to stand until clear and a very slight precipitate has settled; filter through tightly packed acid-washed glass wool. Dilute 10 ml of this approximately 3 N solution to 100 ml with iron-free distilled water; titrate against standard 0.1 N silver nitrate using ferric alum as indicator; adjust accordingly and re-titrate—30 ml of 0.1 N silver nitrate require 10 ml of a 1:10 dilution of 3 N thiocyanate. Discard if any trace of discoloration appears; deterioration is also evidenced by a rapid formation of a yellow color in the blank.

Technic

1. In a 50 ml volumetric flask (sample) place
   0.5 ml oxalated whole blood delivered from an Ostwald-Van Slyke pipette
   2 ml iron-free distilled water (1).
2. Rapidly swirl the flask until the blood is completely hemolyzed (about 2 minutes).
3. Add 2 ml concentrated sulfuric acid (2), drop by drop, while rapidly rotating the flask.
4. Agitate the flask vigorously for 5 minutes and allow to stand for 60 minutes.
5. Add 2 ml saturated potassium persulfate (3), again agitate the flask for 2 minutes and allow to stand for 30 minutes.
6. Add 25 ml iron-free distilled water (1) and 2 ml 10% sodium tungstate (4), while rapidly rotating the flask.
7. Allow to stand for 30 minutes; cool to room temperature under running tap water if necessary.
8. Carefully, so as to prevent foaming, dilute to 50 ml with iron-free distilled water (1) and mix thoroughly by repeated inversion.

9. In another 50 ml volumetric flask (reagent blank) place
   25 ml iron-free distilled water (1)
   2 ml concentrated sulfuric acid (2)
   2 ml saturated potassium persulfate (3), mix, cool and dilute to 50 ml with iron-free distilled water.
10. Filter the contents of each flask through identical acid-washed Whatman #40 papers into correspondingly marked dry 125 ml Erlenmeyer flasks, refiltering the first few drops if necessary.
11. Transfer 10 ml of each filtrate to a correspondingly marked accurately calibrated 25 ml glass-stoppered cylinder.
12. To each cylinder add
    1 ml saturated potassium persulfate (3)
    2 ml 3 N potassium thiocyanate (5) and mix by lateral shaking.

Note: The color is light sensitive and after development, undue exposure to direct light should be avoided.

13. Dilute to 20 ml with iron-free distilled water (1), stopper and mix by inversion.
14. Measure the transmittancy of the sample against the reagent blank, which should be colorless, set at 100% transmission at a wave length of 480 mμ and obtain the concentration of iron from the calibration table.

CALIBRATION PROCEDURE

Special Apparatus

Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.
Graduated glass-stoppered cylinders—25 ml; the 20 ml graduation must be accurately calibrated.

Reagents

1. **Water, Distilled, Iron-Free.** Same as for Test Procedure.
2. **Sulfuric Acid, Concentrated, Iron-Free.** Same as for Test Procedure.
3. **Potassium Persulfate, Saturated Aqueous.** Same as for Test Procedure.
5. **Potassium Thiocyanate, Volumetric 3 N.** Same as for Test Procedure.
6. **Nitric Acid, Concentrated, Iron-Free** (HNO_3—Grasselli, A.C.S.).
7. **Iron Standards**
   (a) Iron stock standard, Dupray (1 ml contains 1 mg ferric iron). Thoroughly clean a small piece of iron wire of known iron content (Special for Standardization, usually 99.85% Fe—Mallinckrodt, AR) with fine emery cloth or paper until shining and free of rust and dirt. Knowing the iron content, weigh out an amount of wire that contains 100 mg of iron. In a 250 ml Florence flask place 10 ml concentrated iron-free nitric acid (6) and approximately 40 ml iron-free distilled water (1); heat to boiling; roll the wire into a loose coil and drop, together with any fine trimmings, into the boiling acid; boil until completely dissolved and then one minute longer. Cool and transfer quantitatively to a 100 ml volumetric flask; dilute to volume with iron-free distilled water and mix. Stable indefinitely.
   (b) Dilute standard (1 ml contains 0.1 mg ferric iron). In a 100 ml volumetric flask place 10 ml stock standard (7a) and dilute to volume with iron-free distilled water (1). Prepare day of use.
   (c) Calibration standards. In a series of seven accurately calibrated 50 ml volumetric flasks place respectively 0, 1, 1.5, 2, 2.5, 3 and 4 ml of the dilute standard (7b); add 2 ml concentrated sulfuric acid (2), and 2 ml saturated potassium persulfate (3); dilute to volume with iron-free distilled water (1). These standards represent concentrations of iron equivalent to 0, 20, 30, 40, 50, 60 and 80 mg per 100 ml sample. Prepare just before use.

Technic

1. Transfer 10 ml of each calibration standard (7c) to a correspondingly marked accurately calibrated 25 ml glass-stoppered cylinder.
2. Proceed exactly as outlined in the Test Procedure, Steps 12–13 inclusive.
3. Set the zero concentration standard at 100% transmission at a wave length of 480 mμ and record the transmittancy of the 20, 30, 40, 50, 60 and 80 mg per cent standards. The transmittancy of the 80 mg standard should be approximately 23%.
4. Plot the observed values on semi-log graph paper—the points fall on a straight line which passes through the origin (Calibration Curve I-28).
5. Prepare a calibration table from the T-C values obtained from the plotted curve.

CONVERSION FACTORS

To convert iron in mg per 100 ml to hemoglobin in gm per 100 ml divide by 3.35 (Butterfield).
To convert iron in mg per 100 ml to oxygen capacity in volumes per cent multiply by 0.4 (Van Slyke).

PROCEDURE FOR USE WITH LINEAR-SCALE PHOTOELECTRIC COLORIMETERS (SHEARD-SANFORD TYPE)

TEST PROCEDURE

Exactly as outlined; set the reagent blank at 100 and use a green filter.

CALIBRATION PROCEDURE

Exactly as outlined—use a green filter; plot the observed readings on semi-log graph paper—the points fall on a straight line passing through the origin.

PROCEDURE FOR USE WITH LOGARITHMIC-SCALE PHOTOELECTRIC COLORIMETERS (KLETT-SUMMERSON TYPE)

TEST PROCEDURE

Exactly as outlined; set the reagent blank at 0 and use a green filter.

CALIBRATION PROCEDURE

Exactly as outlined—use a green filter; plot the observed readings on linear graph paper—the points fall on a straight line passing through the origin.

BIBLIOGRAPHY

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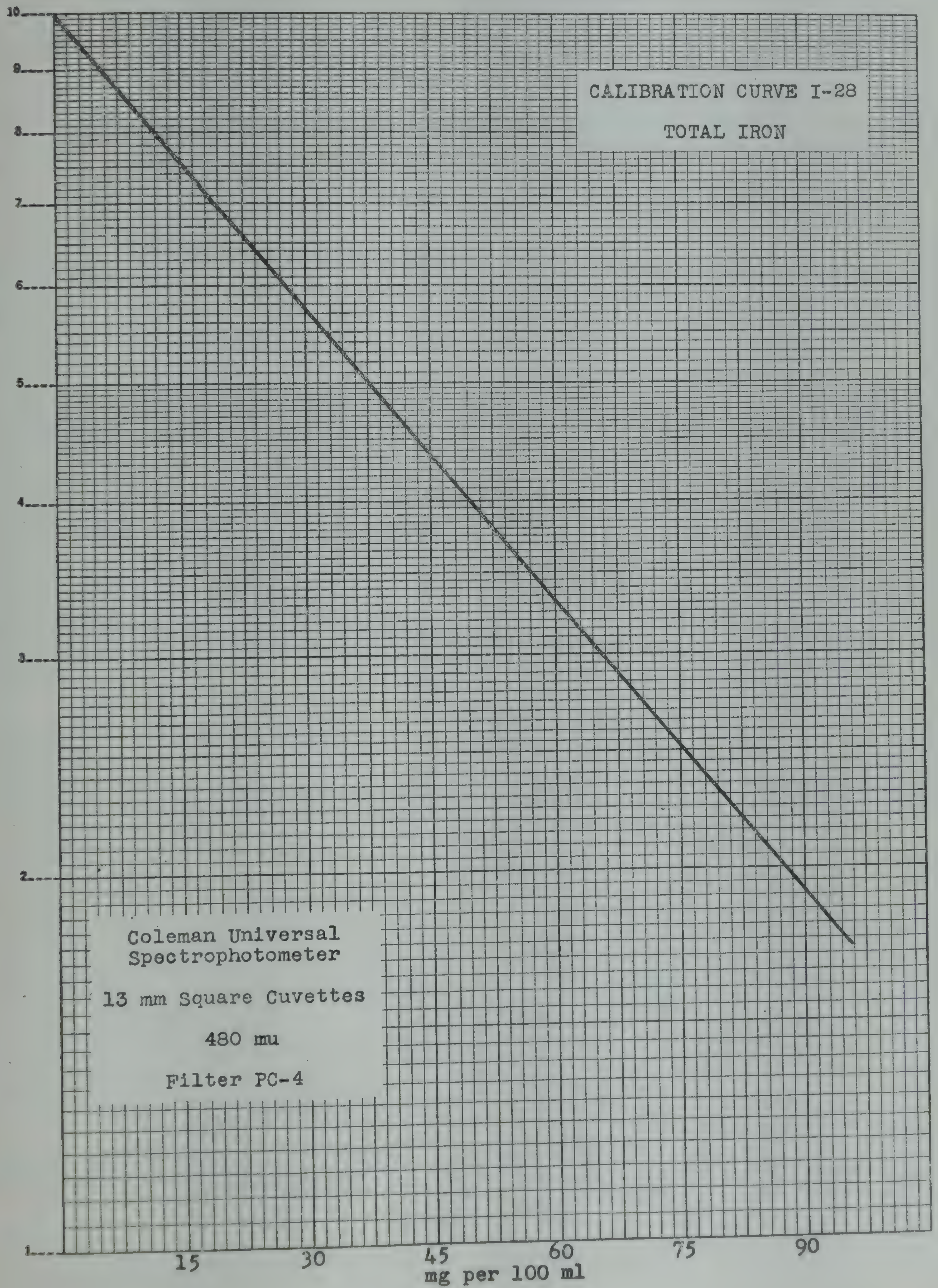
CALIBRATION CURVE I-28
TOTAL IRON
10
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Coleman Universal Spectrophotometer
13 mm Square Cuvettes
480 mu
Filter PC-4
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mg per 100 ml

Meter Reading	0	1	2	3	4	5	6	7	8	9
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17-KETOSTEROIDS, TOTAL

A spectrophotometric adaptation based on the methods and modifications of Zimmerman (1935; 1936), Girard and Sandulesco (1936), Gallagher, Peterson, Dorfman, Kenyon and Koch (1937), Callow, Callow and Emmens (1938), Butler and Marrian (1938), Langstroth and Talbot (1939), Talbot and Langstroth (1939), Talbot, Butler and MacLachlan (1940), Holtorff and Koch (1940), Talbot, Butler, MacLachlan and Jones (1940), Pincus and Pearlman (1941), Koch (1942), Talbot, Berman and MacLachlan (1942), Nathanson and Wilson (1943), Wilson and Nathanson (1945) and Landau (1949)

PRINCIPLE

Urine is subjected to hydrochloric acid hydrolysis to convert the conjugated steroids to the free form. The hydrolyzed steroids together with the free steroids originally present and numerous other compounds are then extracted by ether. Most of the impurities are removed by subsequent washings of the extract with sodium bicarbonate and sodium hydroxide. Some non-ketonic chromogens still remain in the extract—for routine purposes, their removal is not necessary and Procedure A is followed; however, for more precise work, Procedure B is used in which the ketonic substances are separated by means of Girard's Reagent T. In both cases, the extract is evaporated to dryness, taken up in alcohol and treated with m-dinitrobenzene in the presence of potassium hydroxide to develop a red color (Zimmerman reaction). Androsterone and dehydroisoandrosterone give practically identical color values.

DETERMINATION IN URINE

SAMPLE

A 500 ml aliquot of the total 24-hour specimen. Keep cool but use no preservative.

TEST PROCEDURE

Special Apparatus

Florence flask, 1000 ml, Pyrex, with ground glass joint and reflux condenser to fit.

Separatory funnels, 500 ml and 1000 ml; pear-shaped.

Boiling flasks, 200 ml, round-bottom with ground glass joints and reflux condensers to fit.

Micro burettes—2 ml in 0.01 ml (micro pipettes accurately calibrated "to deliver" 0.2 ml may be used but are less convenient).

Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.

Note: Glassware must be chemically clean. Stopcock grease or oil must not be used. All joints must be ground glass—the use of corks or rubber connections is not permissible.

Reagents

1. **Hydrochloric Acid, Concentrated** (HCl—Baker & Adamson, A.C.S.).
2. **Ether, Anhydrous, Peroxide-Free** ($(C_2H_5)_2O$—Mallinckrodt, AR). All samples of ether should be checked for the presence of peroxide as follows: Shake a small sample of ether with one-half volume freshly prepared 10% potassium iodide slightly acidified with concentrated sulfuric acid; if more than a very faint yellow color develops, the ether must be purified by washing with 10% sodium sulfite followed by several washings with distilled water, and distilled over calcium chloride. Preserve in the dark in a tightly stoppered brown glass bottle containing a coil of pure iron wire.
3. **Sodium Bicarbonate, Saturated Aqueous.** In a 2000 ml beaker heat 1000 ml distilled water to 60° C.; add 150 gm sodium bicarbonate ($NaHCO_3$—Baker & Adamson, A.C.S.) and stir until dissolved; allow to cool and sediment; transfer the clear supernatant fluid to a ceresin-lined bottle.
4. **Sodium Hydroxide, Volumetric 2.5 N.** Prepare by diluting slightly more than the required amount of concentrated carbonate-free sodium hydroxide solution (see Appendix); dilute 20 ml of this approximately 2.5 N solution to 100 ml; titrate against standard 0.5 N acid using phenolphthalein as indicator; adjust accordingly and re-titrate—20 ml of 0.5 N acid require 20 ml of a 1:5 dilution of 2.5 N alkali. Preserve in ceresin-lined bottle.
5. **Sulfuric Acid, Volumetric 2 N.** Slowly add 60 ml concentrated sulfuric acid (H_2SO_4—Grasselli, A.C.S.) to 1000 ml distilled water and mix well; allow to cool; dilute 10 ml of this approximately 2 N solution to 100 ml; titrate against standard 0.1 N alkali using phenolphthalein as indicator; adjust accordingly and re-titrate—10 ml of a 1:10 dilution of 2 N acid require 20 ml of 0.1 N alkali.
6. **Ethyl Alcohol, Absolute** (C_2H_5OH—U. S. Industrial Chemicals, U.S.P.). Should be re-distilled frequently.
7. **Potassium Hydroxide, Volumetric 5 N.** In a 2000 ml Florence flask place 700 ml distilled water; add slowly and with constant shaking, 350 gm potassium hydroxide pellets (KOH—Baker & Adamson, Reagent Special, Low Carbonate); shake until dissolved and allow to stand until cool; after sedimentation is complete, dilute 2 ml of this approximately 5 N clear supernatant fluid to 100 ml; titrate against standard 0.1 N acid

using phenolphthalein as indicator; adjust accordingly and re-titrate—20 ml of 0.1 N acid require 20 ml of a 1:50 dilution of 5 N alkali. The normality of this solution must be accurate to ±0.02 N. Prepare fresh monthly. Preserve in ceresin-lined bottle in refrigerator.

Note: Check carbonate content of each lot of potassium hydroxide by adding 10.4 ml 95% ethyl alcohol (10) to 0.2 ml of the 5 N hydroxide—this solution must remain perfectly clear; otherwise prepare fresh 5 N solution from another lot of hydroxide. Turbidity by this test indicates that a similar turbidity will be encountered in the final dilution and thus affect the accuracy of the measurements.

8. **m-Dinitrobenzene, 2% Alcoholic.** In a 100 ml mixing cylinder place 2 gm m-dinitrobenzene ($C_6H_4(NO_2)_2$—Reagent Chemicals, Special Recrystallized); add absolute ethyl alcohol (6) to volume; shake until dissolved. Preserve in a brown glass bottle in refrigerator. Prepare fresh every 10 days or sooner, if a discoloration appears.
9. **Ethyl Alcohol, 60% Aqueous.** Dilute 60 ml absolute ethyl alcohol (6) to 100 ml with distilled water; allow to cool and adjust volume.
10. **Ethyl Alcohol, 95%** (C_2H_5OH—U. S. Industrial Chemicals, U.S.P.).
11. **Acetic Acid, Glacial** (CH_3COOH—Baker & Adamson, A.C.S.).
12. **Girard's Reagent T** ($(CH_3)_3N(Cl)CH_2CONHNH_2$—Matheson #5912, Trimethylacethydrazide ammonium chloride).
13. **Sulfuric Acid, Concentrated** (H_2SO_4—Grasselli, A.C.S.).

Technic

Note: Precautions should be taken to prevent loss by foaming or bumping during the evaporation of the solvents.

A. Without Separation of Non-Ketonic Substances

Note: For concentrations of total 17-ketosteroids less than 0.5 mg per 100 ml, use a 10 ml volumetric flask in Step 27 and multiply the values obtained from the calibration table by 0.4; for concentrations in excess of 2.5 mg per 100 ml, use a 50 ml volumetric flask in Step 27 and multiply the values by 2.

1. In a 1000 ml Florence flask with ground glass joint place a 500 ml aliquot of thoroughly mixed urine.
2. Add 50 ml concentrated hydrochloric acid (1) and mix thoroughly.
3. Connect a reflux condenser and boil for 15 minutes.
4. Disconnect condenser and rapidly cool the flask under running water to room temperature.
5. Quantitatively transfer the hydrolysate to a 1000 ml pear-shaped separatory funnel, washing out the flask with a minimum quantity of distilled water.
6. Immediately add 75 ml peroxide-free ether (2), stopper the funnel and shake vigorously for 3 minutes.
7. Allow to stand until the layers separate—about 2 minutes.
8. Transfer the lower aqueous phase to another 1000 ml separatory funnel—reserve the ether phase for Step 11.
9. Add 60 ml peroxide-free ether (2), shake vigorously for 3 minutes and allow to stand until the layers separate.
10. Draw off and discard the lower aqueous phase.
11. Add the ether extract in the first funnel to the extract in the second, washing out the first funnel with two 10 ml portions peroxide-free ether (2) and transferring the washings to the combined extracts.
12. Add 40 ml saturated sodium bicarbonate (3) to the combined extracts and wash by gentle shaking, avoiding any loss due to foaming; occasionally an emulsion may occur at this point—it may be broken by the addition of 25 ml 2.5 N sodium hydroxide (4).
13. Allow the layers to separate (about 2 minutes); draw off and discard the lower aqueous phase.
14. Quantitatively transfer the ether extract to a 500 ml separatory funnel.
15. Add 40 ml saturated sodium bicarbonate (3), shake gently, allow the layers to separate and discard the lower aqueous phase.
16. Repeat Step 15.
17. Add 20 ml 2.5 N sodium hydroxide (4), shake gently, allow the layers to separate and discard the lower aqueous phase.
18. Repeat Step 17 three times.
19. Add 15 ml 2 N sulfuric acid (5), shake thoroughly, allow the layers to separate and discard the lower aqueous phase.
20. Add 25 ml distilled water, shake thoroughly, allow the layers to separate (about 2 minutes) and discard the lower aqueous phase.
21. Repeat Step 20 twice, allowing the layers to separate about 5 minutes the first time and 10 minutes the second time.
22. Quantitatively transfer the ether extract to a 200 ml round-bottom flask.
23. Place the flask in a hot water bath and carefully evaporate to dryness under reduced pressure.
24. Add 30 ml absolute ethyl alcohol (6) and agitate the flask until the residue is completely dissolved.
25. Repeat Step 23.
26. Add 5 ml absolute ethyl alcohol (6) and gently agitate the flask to completely dissolve the residue—gentle heat may be used if necessary to aid solution.
27. Quantitatively transfer the contents of the flask to a 25 ml volumetric flask and dilute to volume with repeated absolute ethyl alcohol (6) washings of the round-bottom flask.
28. In a cuvette (sample) place
    0.2 ml 5 N potassium hydroxide (7)
    0.2 ml 2% m-dinitrobenzene (8)

0.2 ml alcoholic solution of the extract from Step 27 and mix thoroughly.

29. In another cuvette (sample blank) place
    - 0.2 ml 5 N potassium hydroxide (7)
    - 0.2 ml absolute ethyl alcohol (6)
    - 0.2 ml alcoholic solution of the extract from Step 27 and mix thoroughly.
30. Stopper each cuvette with a clean cork covered with aluminum foil and place in a water bath at 25° C. (±0.2° C.) for 105 minutes—during this color development period, the cuvettes must not be exposed to light and the water bath should be kept covered.
31. Remove the cuvettes from the bath and immediately add 10 ml 60% ethyl alcohol (9) and mix.
32. Allow to stand in the dark at room temperature for 3 minutes.
33. Within 7 minutes, measure the transmittancy of the sample against the sample blank set at 100% transmission at a wave length of 520 mμ and obtain the concentration of total 17-ketosteroids from the calibration table.

Calculation

$$\frac{\text{Volume of 24-hour specimen}}{100} \times \text{Total 17-ketosteroids in mg per 100 ml} = \text{Mg total 17-ketosteroids excreted in 24 hours}$$

Method K-10b

B. With Separation of Non-Ketonic Substances

Note: For concentrations of total 17-ketosteroids less than 0.5 mg or greater than 2.5 mg per 100 ml, dilute the final extract to 10 ml or 50 ml respectively—see note under Procedure A.

1. Proceed exactly as outlined in Procedure A, Steps 1–18 inclusive.
2. Add 20 ml distilled water, shake thoroughly, allow the layers to separate (about 2 minutes) and discard the lower aqueous phase.
3. Repeat Step 2 twice, allowing the layers to separate about 5 minutes the first time and 10 minutes the second time.
4. Quantitatively transfer the ether extract to a 200 ml round-bottom flask with ground glass joint.
5. Place the flask in a hot water bath and carefully evaporate to dryness under reduced pressure.

Note: Carry out Steps 6–23 inclusive as rapidly as possible.

6. Allow to cool and add
    - 4 ml 95% ethyl alcohol (10) and agitate the flask until the residue is completely dissolved
    - 0.5 ml glacial acetic acid (11)
    - 0.5 gm Girard's Reagent T (12).
7. Connect condenser, place the flask in a boiling water bath and reflux for 1 hour.
8. Allow to cool to room temperature and disconnect condenser.
9. Quantitatively transfer the contents of the flask to a 500 ml separatory funnel, washing out the flask with repeated portions of ice water until a total of 40 ml has been used.
10. Immediately add 3 ml 2.5 N sodium hydroxide (4) and mix.
11. Add 40 ml peroxide-free ether (2), stopper the funnel and shake vigorously for 3 minutes.
12. Allow to stand until the layers separate.
13. Transfer the lower aqueous phase to another 500 ml separatory funnel—reserve the ether phase.
14. Add 40 ml peroxide-free ether (2) to the funnel containing the aqueous phase, shake vigorously and repeat Steps 12–13 inclusive, combining the ether phases in one funnel—reserve the aqueous phase for Step 16.
15. Repeat Step 14 twice.
16. To the combined ether extracts add 20 ml ice-cold distilled water; shake thoroughly, allow the layers to separate (about 2 minutes) and draw off the lower layer into the funnel containing the aqueous phases from Steps 14 and 15—discard the ether extract.
17. Repeat Step 16 twice, allowing the layers to separate about 5 minutes the first time and 10 minutes the second time, combining the aqueous phases in the one funnel.
18. To the funnel containing the aqueous phases add 1 ml concentrated sulfuric acid (13) and 20 ml peroxide-free ether (2).
19. Shake thoroughly and allow to stand at room temperature for 2 hours.
20. Add 1 ml concentrated sulfuric acid (13) and mix.
21. Add 40 ml peroxide-free ether (2), shake vigorously for 3 minutes, allow the layers to separate, draw off the lower aqueous phase into another 500 ml separatory funnel—reserve the ether phase.
22. Add 40 ml peroxide-free ether (2) to the funnel containing the aqueous phase and repeat Step 21, combining the ether extracts in one funnel.
23. Repeat Step 22 twice.
24. To the combined ether extracts add 20 ml distilled water, shake, allow the layers to separate (about 2 minutes), draw off and discard the lower aqueous phase.
25. Repeat Step 24 twice, allowing the layers to separate about 5 minutes the first time and 10 minutes the second time.
26. Quantitatively transfer the ether extract to a 200 ml round-bottom flask, washing out the funnel with several small portions peroxide-free ether (2).

27. Proceed exactly as outlined in Procedure A, Steps 23–27 inclusive.
28. In a cuvette (sample) place
    0.2 ml 5 N potassium hydroxide (7)
    0.2 ml 2% m-dinitrobenzene (8)
    0.2 ml alcoholic solution of the extract from Step 27 and mix thoroughly.
29. In another cuvette (reagent blank) place
    0.2 ml 5 N potassium hydroxide (7)
    0.2 ml 2% m-dinitrobenzene (8)
    0.2 ml absolute ethyl alcohol (6) and mix.
30. Stopper each cuvette with a clean cork covered with aluminum foil and place in a water bath at 25° C. (±0.2° C.) for 105 minutes—during this color development period, the cuvettes must not be exposed to light and the water bath should be kept covered.
31. Remove the cuvettes from the bath and immediately add 10 ml 60% ethyl alcohol (9) and mix.
32. Allow to stand in the dark at room temperature for 3 minutes.
33. Within 7 minutes, measure the transmittancy of the sample against the reagent blank set at 100% transmission at a wave length of **520 mμ** and obtain the concentration of total 17-ketosteroids from the calibration table.

Calculation

Same as under Procedure A.

CALIBRATION PROCEDURE

Special Apparatus

Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.
Micro burettes—2 ml in 0.01 ml.
Micro pipettes accurately calibrated "to deliver" 0.2 ml.

Reagents

6. **Ethyl Alcohol, Absolute.** Same as for Test Procedure.
7. **Potassium Hydroxide, Volumetric 5 N.** Same as for Test Procedure.
8. **m-Dinitrobenzene, 2% Alcoholic.** Same as for Test Procedure.
9. **Ethyl Alcohol, 60% Aqueous.** Same as for Test Procedure.
14. **17-Ketosteroid Standards**
    (a) Dehydroisoandrosterone stock standard (1 ml contains 1 mg dehydroisoandrosterone). In a 50 ml volumetric flask place 57.5 mg dehydroisoandrosterone acetate ($CH_3COO\text{-}C_{19}H_{27}O$—Reagent Chemicals, Special); add approximately 40 ml absolute ethyl alcohol (6); shake until dissolved and dilute to volume with same. Preserve in refrigerator.
    (b) Calibration standards. In a series of six accurately calibrated 10 ml volumetric flasks place respectively 0, 1, 2, 3, 4 and 5 ml of the stock standard (14a) and dilute to volume with absolute ethyl alcohol (6). These standards represent concentrations of 17-ketosteroids equivalent to 0, 0.5, 1.0, 1.5, 2.0 and 2.5 mg per 100 ml sample. Prepare just before use.

Technic

1. In a series of six correspondingly marked cuvettes place
    0.2 ml 5 N potassium hydroxide (7)
    0.2 ml 2% m-dinitrobenzene (8) and mix.
2. Transfer 0.2 ml of each calibration standard (14b), by means of a micro pipette, to the proper cuvette and mix.
3. Proceed exactly as outlined in Test Procedure A, Steps 30–32 inclusive.
4. Within 7 minutes set the zero concentration standard at 100% transmission at a wave length of **520 mμ** and record the transmittancy of the 0.5, 1.0, 1.5, 2.0 and 2.5 mg per cent standards, completing all readings within 10 minutes. The transmittancy of the 2.5 mg standard should be approximately 26%.
5. Plot the observed values on semi-log graph paper—the points fall on a straight line which passes through the origin (Calibration Curve K-10).
6. Prepare a calibration table from the T-C values obtained from the plotted curve.

PROCEDURE FOR USE WITH LINEAR-SCALE PHOTOELECTRIC COLORIMETERS (SHEARD-SANFORD-TYPE)

TEST PROCEDURE

Exactly as outlined; set the blanks at 100 and use a green filter.

CALIBRATION PROCEDURE

Exactly as outlined—use a green filter; plot the observed readings on semi-log graph paper—the points fall on a straight line passing through the origin.

PROCEDURE FOR USE WITH LOGARITHMIC-SCALE PHOTOELECTRIC COLORIMETERS (KLETT-SUMMERSON TYPE)

TEST PROCEDURE

Exactly as outlined; set the blanks at 0 and use a green filter.

CALIBRATION PROCEDURE

Exactly as outlined—use a green filter; plot the observed readings on linear graph paper—the points fall on a straight line passing through the origin.

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NOTES

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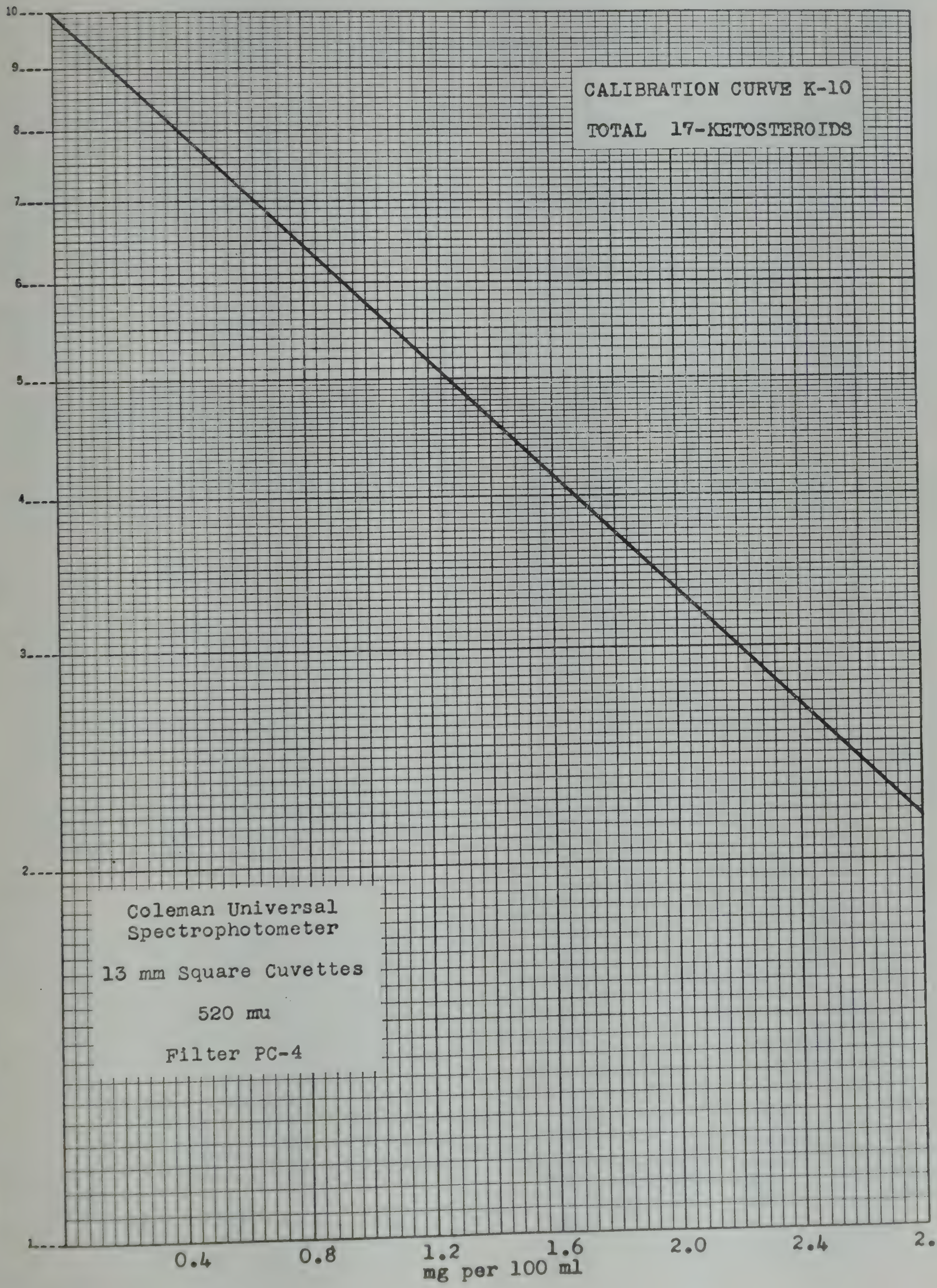
CALIBRATION CURVE K-10
TOTAL 17-KETOSTEROIDS
Coleman Universal Spectrophotometer
13 mm Square Cuvettes
520 mu
Filter PC-4
10
9
8
7
6
5
4
3
2
1
0.4
0.8
1.2
1.6
2.0
2.4
2.8
mg per 100 ml

Meter Reading	0	1	2	3	4	5	6	7	8	9
0										
10										
20										
30										
40										
50										
60										
70										
80										
90										

LACTIC ACID

A spectrophotometric adaptation based on the methods and modifications of
Van Slyke (1917), Dische and Laszlo (1927) and Hoffman (1941)

PRINCIPLE

The protein-free filtrate is treated with copper sulfate and calcium hydroxide to remove dextrose and other interfering substances. Sulfuric acid converts lactic acid to acetaldehyde which is then treated with hydroquinone to produce a condensation product with an orange yellow color.

DETERMINATION IN WHOLE BLOOD, PLASMA OR SERUM

SAMPLE

Blood withdrawn without stasis from resting subject during post-absorptive state. When analyzing whole blood or plasma, potassium oxalate may be used as an anticoagulant—if the proteins cannot be precipitated immediately after withdrawl of the blood add 2 mg ammonium fluoride for each ml of blood and store in refrigerator until use.

TEST PROCEDURE

Special Apparatus

Centrifuge tubes, 15 ml, glass-stoppered.

Ostwald-Folin pipettes accurately calibrated "to contain" 1 ml.

Graduated test tubes—25 ml in 0.5 ml; the 10 ml graduation must be accurately calibrated.

Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.

Note: All apparatus must be scrupulously cleaned with hot sulfuric acid, rinsed with distilled water and oven dried before use. Dry glassware must be used throughout the procedure. Do not use dichromate-sulfuric acid solution for cleaning or acetone for quickly drying glassware.

Reagents

1. **Metaphosphoric Acid, 6% Aqueous.** Using only clear pellets, dissolve 6 gm glacial metaphosphoric acid (HPO_3—Baker & Adamson, Reagent) in distilled water and dilute to 100 ml. Although this reagent remains stable for 1–2 weeks in the refrigerator, it is better to prepare small amounts fresh each day. Do not filter.
2. **Copper Sulfate, 15% Aqueous.** Dissolve 15 gm crystalline copper sulfate ($CuSO_4 \cdot 5H_2O$—Baker & Adamson, A.C.S., Fine Crystals) in distilled water and dilute to 100 ml. Do not filter.
3. **Calcium Hydroxide, Powder** ($Ca(OH)_2$—Mallinckrodt, AR).
4. **Sulfuric Acid, Concentrated** (H_2SO_4—Grasselli, A.C.S.). Adequate precautions should be taken to prevent contamination by organic dust and moisture. Avoid undue exposure to air. The acid must be perfectly colorless. A separate bottle of the acid should be reserved for lactic acid determinations—pour out the amount of acid as required—do not contaminate by pipetting; discard excess acid—do not pour back into stock bottle.
5. **Hydroquinone, 20% Alcoholic.** In a 10 ml mixing cylinder place 5 ml 95% ethyl alcohol (C_2H_5OH—U.S. Industrial Chemicals, U.S.P.); add 1 gm white hydroquinone crystals (C_6H_4-1,4-$(OH)_2$—Eastman #356) and shake until dissolved. A slight opacity which sometimes appears may be disregarded. This solution must be prepared fresh daily, preferably just before use.

Technic

Note: Those portions of the glassware which come into contact with the reagents should not be handled. When shaking or mixing by inversion, glass-stoppered tubes must be used—never use the finger or the palm of the hand as this introduces a considerable positive error. At no point is filtration permissible.

For concentrations of lactic acid in excess of 75 mg per 100 ml, use 2 ml protein-free centrifugate and 2 ml distilled water in Step 7 and multiply the values obtained from the calibration table by 2.

1. In a 15 ml glass-stoppered centrifuge tube (sample) place
   6 ml distilled water
   1 ml sample from an Ostwald-Folin pipette, thoroughly rinsing out the pipette by drawing in and expelling some of the mixture several times.
2. Rotate the tube between the hands until the blood is completely laked.
3. In another 15 ml glass-stoppered centrifuge tube (reagent blank) place
   7 ml distilled water.
4. To each tube add 1 ml 6% metaphosphoric acid (1).
5. Stopper the tubes, shake vigorously for 30 seconds and allow to stand for 5 minutes.
6. Centrifuge at 1500 rpm for 5 minutes—do not filter.
7. Transfer 4 ml of each clear centrifugate to a correspondingly marked 15 ml glass-stoppered centrifuge tube.
8. To each tube add 1 ml 15% copper sulfate (2) and mix.
9. Add approximately 1 gm calcium hydroxide (3), stopper and shake vigorously for 30 seconds—if the mixture is not bright blue in color, add more hydroxide.
10. Allow to stand for 30 minutes, repeating the shaking at 10 minute intervals.

11. Centrifuge at 1500 rpm for 5 minutes—do not filter.
12. Fill a 400 ml beaker with cracked ice and place two correspondingly marked accurately graduated 25 ml test tubes in an upright position in the ice bath.
13. Transfer 1 ml of each clear centrifugate to the correspondingly marked 25 ml test tube in the ice bath—this should be done by inserting the tip of the pipette beneath the surface of the fluid, holding the index finger over the top of the pipette to prevent the ingress of any of the surface film; after withdrawal of the pipette, wipe off any adherent particles with clean tissue.
14. Without removing the tubes from the ice bath, add 3 ml concentrated sulfuric acid (4), dropwise from a burette, constantly agitating the tubes until the components are thoroughly mixed.

Note: Do not use stop-cock grease on the burette—lubricate with sulfuric acid. Precautions should be taken to avoid absorption of moisture by the acid.

15. To the tubes in the ice bath add 0.1 ml 20% hydroquinone (5), allowing the reagent to fall directly into the acid, agitating constantly—adequate mixing is essential.
16. Remove the tubes from the ice bath and mix contents by vigorous lateral shaking.
17. Place the tubes in vigorously boiling water for 20 minutes—the level of the fluid in the tubes must be below the level of the water in the bath.
18. Transfer the tubes to the ice bath and allow to remain for 2 minutes.
19. Without removing the tubes from the ice bath, add 5 ml distilled water, mixing thoroughly by constant agitation.
20. Allow the tubes to remain in the ice bath for 3 minutes.
21. Remove the tubes from the ice bath and allow to stand at room temperature for 10 minutes—the solutions must be free of gas bubbles.
22. Dilute to 10 ml with distilled water and mix by lateral shaking.
23. Measure the transmittancy of the sample against the reagent blank set at 100% transmission at a wave length of 420 mμ and obtain the concentration of lactic acid from the calibration table.

CALIBRATION PROCEDURE

Special Apparatus

Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.
Graduated test tubes—25 ml in 0.5 ml; the 10 ml graduation must be accurately calibrated.

Reagents

4. **Sulfuric Acid, Concentrated.** Same as for Test Procedure.
5. **Hydroquinone, 20% Alcoholic.** Same as for Test Procedure.
6. **Lactic Acid Standards**
   (a) Lactic acid stock standard (1 ml contains 1 mg lactic acid). In a 100 ml volumetric flask place 106.6 mg recrystallized lithium lactate ($LiC_3H_5O_3$—Reagent Chemicals, Special); add approximately 80 ml distilled water and shake until dissolved; add 2 drops concentrated sulfuric acid (4) and dilute to volume. Preserve in refrigerator. Stability indefinite.
   (b) Dilute standard (1 ml contains 0.15 mg lactic acid). In a 100 ml volumetric flask place 15 ml stock standard (6a) and dilute to volume with distilled water. Prepare just before use.
   (c) Calibration standards. In a series of six accurately calibrated 10 ml volumetric flasks place, respectively, 0, 1, 2, 3, 4 and 5 ml of the dilute standard (6b) and dilute to volume with distilled water. These standards represent concentrations of lactic acid equivalent to 0, 15, 30, 45, 60 and 75 mg per 100 ml sample. Prepare just before use and proceed immediately as follows.

Technic

1. Fill a 1000 ml beaker with cracked ice and place six correspondingly marked accurately graduated 25 ml test tubes in an upright position in the ice bath.
2. Transfer 1 ml of each calibration standard (6c) to the correspondingly marked 25 ml test tube in the ice bath.
3. Proceed exactly as outlined in the Test Procedure, Steps 14–22 inclusive.
4. Set the zero concentration standard at 100% transmission at a wave length of 420 mμ and record the transmittancy of the 15, 30, 45, 60 and 75 mg per cent standards. The transmittancy of the 75 mg standard should be approximately 19%.
5. Plot the observed values on semi-log graph paper—the points fall on a very slight curve which passes through the origin (Calibration Curve L-10).
6. Prepare a calibration table from the T-C values obtained from the plotted curve.

Method L-10b

DETERMINATION IN URINE

SAMPLE

Aliquot of a 24-hour specimen. Keep cool during collection.

TEST PROCEDURE

Special Apparatus

As listed under the Test Procedure for blood.

Reagents

1. **Metaphosphoric Acid, 6% Aqueous.** Same as for blood.
2. **Copper Sulfate, 15% Aqueous.** Same as for blood.
3. **Calcium Hydroxide, Powder.** Same as for blood.
4. **Sulfuric Acid, Concentrated.** Same as for blood.
5. **Hydroquinone, 20% Alcoholic.** Same as for blood.

Technic

Note: See remarks under blood.

1. Centrifuge a small portion of thoroughly mixed urine.
2. In a 15 ml glass-stoppered centrifuge tube (sample) place
   6 ml distilled water
   1 ml centrifuged urine from an Ostwald-Folin pipette, thoroughly rinsing out the pipette by drawing in and expelling some of the mixture several times, and mix.
3. Proceed exactly as outlined in the Test Procedure for blood, Steps 3–22 inclusive.
4. Measure the transmittancy of the sample against the reagent blank set at 100% transmission at a wave length of 420 mμ and obtain the concentration of lactic acid directly from the calibration table for blood.

Calculation

$$\frac{\text{Volume of 24-hour specimen}}{100} \times \text{Lactic acid in mg per 100 ml} = \text{Mg lactic acid excreted in 24 hours}$$

PROCEDURE FOR USE WITH LINEAR-SCALE PHOTOELECTRIC COLORIMETERS (SHEARD-SANFORD TYPE)

TEST PROCEDURE

Exactly as outlined; set the reagent blank at 100 and use a blue filter.

CALIBRATION PROCEDURE

Exactly as outlined—use a blue filter; plot the observed readings on semi-log graph paper—the points fall on a slight curve passing through the origin.

PROCEDURE FOR USE WITH LOGARITHMIC-SCALE PHOTOELECTRIC COLORIMETERS (KLETT-SUMMERSON TYPE)

TEST PROCEDURE

Exactly as outlined; set the reagent blank at 0 and use a blue filter.

CALIBRATION PROCEDURE

Exactly as outlined—use a blue filter; plot the observed readings on linear graph paper—the points fall on a slight curve passing through the origin.

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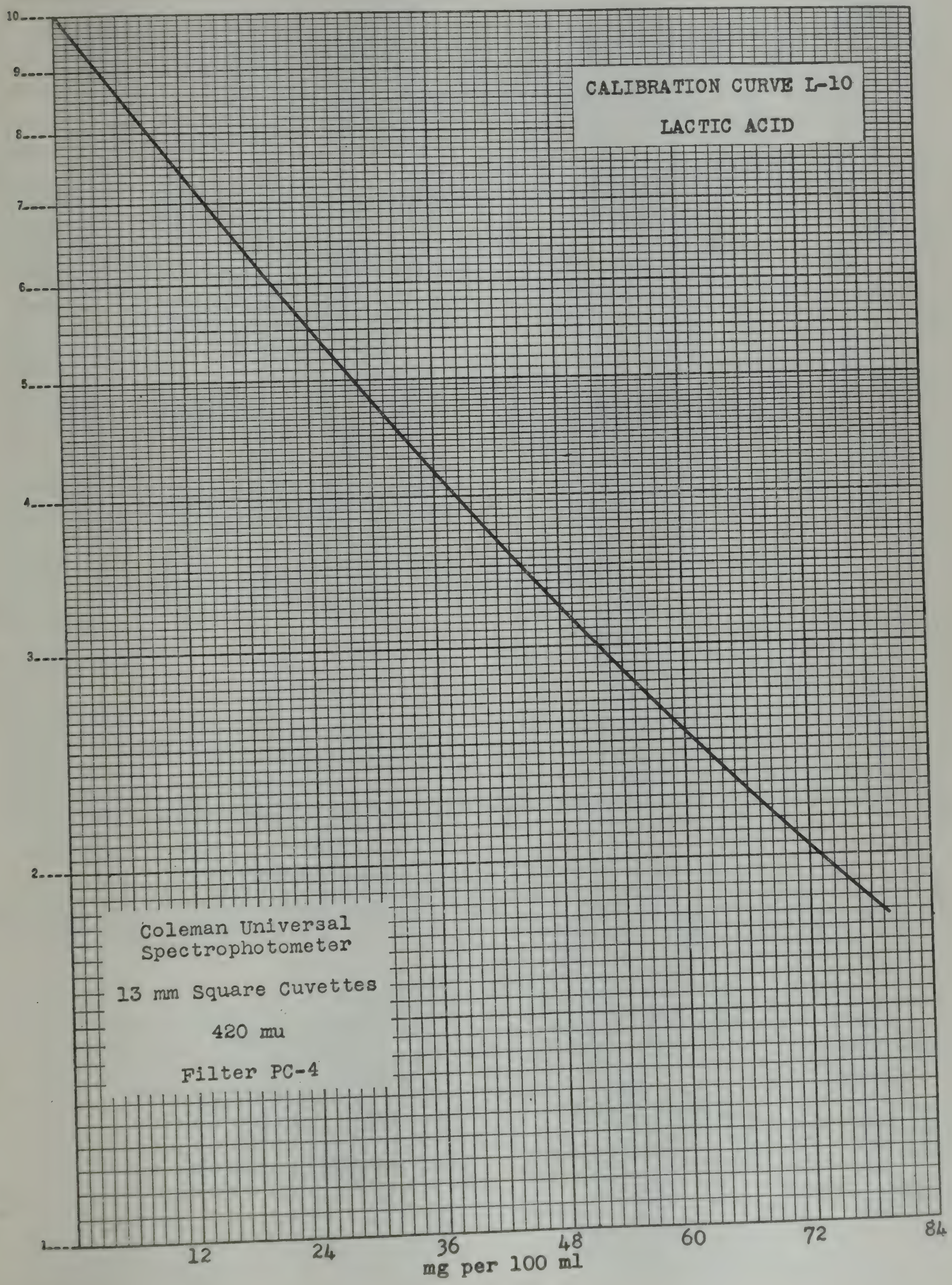
NOTES


CALIBRATION CURVE L-10
LACTIC ACID
Coleman Universal Spectrophotometer
13 mm Square Cuvettes
420 mu
Filter PC-4
10
9
8
7
6
5
4
3
2
1
12
24
36
48
60
72
84
mg per 100 ml

Meter Reading	0	1	2	3	4	5	6	7	8	9
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10										
20										
30										
40										
50										
60										
70										
80										
90										

LACTIC ACID

A spectrophotometric adaptation based on the methods and modifications of Van Slyke (1917), Mendel and Goldscheider (1925), Miller and Muntz (1938), Block and Bolling (1939), Koenemann (1940) and Barker and Summerson (1941)

PRINCIPLE

The protein-free filtrate is treated with copper sulfate and calcium hydroxide to remove dextrose and other interfering substances. Sulfuric acid converts lactic acid to acetaldehyde which is then treated with p-hydroxydiphenyl to produce a condensation product with a purple color (Eegriwe reaction), the intensity of which is enhanced by the addition of copper ions.

DETERMINATION IN WHOLE BLOOD, PLASMA OR SERUM

SAMPLE

Blood withdrawn without stasis from resting subject during post-absorptive state. When analyzing whole blood or plasma, potassium oxalate may be used as an anticoagulant—if the proteins cannot be precipitated immediately after withdrawal of the blood add 2 mg ammonium fluoride for each ml of blood and store in refrigerator until use.

TEST PROCEDURE

Special Apparatus

Graduated centrifuge tubes, 15 ml, glass-stoppered; the 10 ml graduation must be accurately calibrated.

Ostwald-Folin pipettes accurately calibrated "to deliver" 1 ml.

Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.

Note: All apparatus must be scrupulously cleaned with hot sulfuric acid, rinsed with distilled water and oven dried before use. Dry glassware must be used throughout the procedure. Do not use dichromate-sulfuric acid solution for cleaning or acetone for quickly drying glassware.

Reagents

1. **Trichloracetic Acid, 10% Aqueous.** Dilute 10 ml concentrated trichloracetic acid (a) to 100 ml with distilled water. Prepare fresh weekly.
    (a) Trichloracetic acid, concentrated; dissolve 100 gm crystalline trichloracetic acid (CCl_3-$COOH$—J. T. Baker, CP Analyzed) in distilled water and dilute to 100 ml. Preserve in Pyrex glass-stoppered bottle. Stability indefinite.
2. **Copper Sulfate, 20% Aqueous.** Dissolve 20 gm crystalline copper sulfate ($CuSO_4 \cdot 5H_2O$—Baker & Adamson, A.C.S., Fine Crystals) in distilled water and dilute to 100 ml. Do not filter.
3. **Calcium Hydroxide, Powder** ($Ca(OH)_2$—Mallinckrodt, AR).
4. **Copper Sulfate, 4% Aqueous.** Dilute 20 ml 20% copper sulfate (2) to 100 ml with distilled water and mix.
5. **Sulfuric Acid, Concentrated** (H_2SO_4—Grasselli, A.C.S.). Adequate precautions should be taken to prevent contamination by organic dust and moisture. Avoid undue exposure to air.
6. **p-Hydroxydiphenyl Reagent (Barker-Summerson).** In a 250 ml beaker place 1.5 gm p-hydroxydiphenyl ($C_6H_5C_6H_4OH$—Reagent Chemicals, Special, according to Miller and Muntz); add 5 ml 2.5 N sodium hydroxide (a) and 10 ml distilled water; heat until solution is complete, stirring constantly; dilute to 100 ml with distilled water. Preserve in a brown glass bottle. Stability approximately 6 months at room temperature. Discard when the reagent blank shows an appreciable increase.
    (a) Sodium hydroxide, volumetric 2.5 N; prepare by diluting slightly more than the required amount of concentrated carbonate-free sodium hydroxide solution (see Appendix); dilute 20 ml of this approximately 2.5 N solution to 100 ml; titrate against standard 0.5 N acid using phenolphthalein as indicator; adjust accordingly and retitrate—20 ml of 0.5 N acid require 20 ml of a 1:5 dilution of 2.5 N alkali. Preserve in ceresin-lined bottle.

Technic

Note: Those portions of the glassware which come in contact with the reagents should not be handled. When shaking or mixing by inversion, glass-stoppered tubes must be used—never use the finger or the palm of the hand as this introduces a considerable positive error. At no point is filtration permissible.

For concentrations of lactic acid in excess of 75 mg per 100 ml, use 1 ml centrifugate in Step 4 and multiply the values obtained from the calibration table by 2.

1. In a 15 ml glass-stoppered centrifuge tube (sample) place
    9 ml 10% trichloracetic acid (1)
    1 ml sample from an Ostwald-Folin pipette, added drop by drop, while constantly agitating the tube; stopper and shake vigorously for 30 seconds.
2. In another 15 ml glass-stoppered centrifuge tube (reagent blank) place
    9 ml 10% trichloracetic acid (1)
    1 ml distilled water; stopper and mix.

3. Allow to stand for 5 minutes and centrifuge at 1500 rpm for 3 minutes—do not filter.
4. Transfer 2 ml of each clear centrifugate to a correspondingly marked accurately graduated 15 ml glass-stoppered centrifuge tube.
5. To each tube add 1 ml 20% copper sulfate (2) and mix.
6. Dilute to 10 ml with distilled water; stopper and mix.
7. Add approximately 1 gm calcium hydroxide (3), stopper and shake vigorously for 30 seconds—if the mixture is not bright blue in color, add more hydroxide.
8. Allow to stand for 30 minutes, repeating the shaking at 10 minute intervals.
9. Centrifuge at 1500 rpm for 5 minutes—do not filter.
10. Transfer 1 ml of each clear centrifugate to a correspondingly marked 18 × 150 mm Pyrex test tube —this should be done by inserting the tip of the pipette beneath the surface of the fluid, holding the index finger over the top of the pipette to prevent the ingress of any of the surface film; after withdrawal of the pipette, wipe off any adherent particles with clean tissue.
11. To each tube add 0.05 ml 4% copper sulfate (4) and mix—1 drop from a capillary-tipped dropper suffices.
12. Add 8 ml concentrated sulfuric acid (5), dropwise from a burette, constantly agitating the tubes by lateral shaking until the components are thoroughly mixed.

Note: Do not use stop-cock grease on the burette—lubricate with sulfuric acid. Precautions should be taken to avoid absorption of moisture by the acid.

13. Place tubes in boiling water for 5 minutes.
14. Cool under running tap water to at least 20° C.
15. Add 0.1 ml p-hydroxydiphenyl reagent (6), allowing the reagent to fall directly into the acid, while constantly shaking to disperse the precipitate which forms uniformly throughout the solution—adequate mixing is essential.
16. Place the tubes in a water bath at 30° C. for 30 minutes (longer does no harm), redispersing the precipitated reagent by lateral shaking at 10 minute intervals—the solution should now have a violet blue color; if reddish-purple at this stage, it is indicative of too high a temperature and the analysis must be repeated.
17. Place in vigorously boiling water for exactly 90 seconds—the solution should be perfectly clear and reddish-purple in color.
18. Cool to room temperature under running tap water.
19. Measure the transmittancy of the sample against the reagent blank set at 100% transmission at a wave length of 565 mμ and obtain the concentration of lactic acid from the calibration table.

CALIBRATION PROCEDURE

Special Apparatus

Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.

Graduated centrifuge tubes, 15 ml, glass-stoppered; the 10 ml graduation must be accurately calibrated.

Reagents

2. **Copper Sulfate, 20% Aqueous.** Same as for Test Procedure.
3. **Calcium Hydroxide, Powder.** Same as for Test Procedure.
4. **Copper Sulfate, 4% Aqueous.** Same as for Test Procedure.
5. **Sulfuric Acid, Concentrated.** Same as for Test Procedure.
6. **p-Hydroxydiphenyl Reagent (Barker-Summerson).** Same as for Test Procedure.
7. **Lactic Acid Standards**
    - (a) Lactic acid stock standard (1 ml contains 1 mg lactic acid). In a 100 ml volumetric flask place 106.6 mg recrystallized lithium lactate ($LiC_3H_5O_3$—Reagent Chemicals, Special); add approximately 80 ml distilled water and shake until dissolved; add 2 drops concentrated sulfuric acid (5) and dilute to volume. Preserve in refrigerator. Stability indefinite.
    - (b) Dilute standard (1 ml contains 0.03 mg lactic acid). In a 100 ml volumetric flask place 3 ml stock standard (7a) and dilute to volume with distilled water. Prepare just before use.
    - (c) Calibration standards. In a series of six accurately calibrated 10 ml volumetric flasks place respectively 0, 2, 4, 6, 8 and 10 ml of the dilute standard (7b) and dilute to volume with distilled water. These standards represent concentrations of lactic acid equivalent to 0, 15, 30, 45, 60 and 75 mg per 100 ml sample. Prepare just before use and proceed immediately as follows.

Technic

1. Transfer 5 ml of each calibration standard (7c) to a correspondingly marked accurately graduated 15 ml glass-stoppered centrifuge tube.
2. Proceed exactly as outlined in the Test Procedure, Steps 5–18 inclusive.
3. Set the zero concentration standard at 100% transmission at a wave length of 565 mμ and record the transmittancy of the 15, 30, 45, 60 and 75 mg per cent standards. The transmittancy of the 75 mg standard should be approximately 20%.
4. Plot the observed values on semi-log graph paper—the points fall on a very slight curve which passes through the origin (Calibration Curve L-11).

5. Prepare a calibration table from the T-C values obtained from the plotted curve.

PROCEDURE FOR USE WITH LINEAR-SCALE PHOTOELECTRIC COLORIMETERS (SHEARD-SANFORD TYPE)

TEST PROCEDURE

Exactly as outlined; set the reagent blank at 100 and use a green filter.

CALIBRATION PROCEDURE

Exactly as outlined—use a green filter; plot the observed readings on semi-log graph paper—the points fall on a very slight curve passing through the origin.

PROCEDURE FOR USE WITH LOGARITHMIC-SCALE PHOTOELECTRIC COLORIMETERS (KLETT-SUMMERSON TYPE)

TEST PROCEDURE

Exactly as outlined; set the reagent blank at 0 and use a green filter.

CALIBRATION PROCEDURE

Exactly as outlined—use a green filter; plot the observed readings on linear graph paper—the points fall on a very slight curve passing through the origin.

Note: The usual green filters may be employed; however, best results are obtained when filters with a peak transmission of 560–565 mμ are used.

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NOTES

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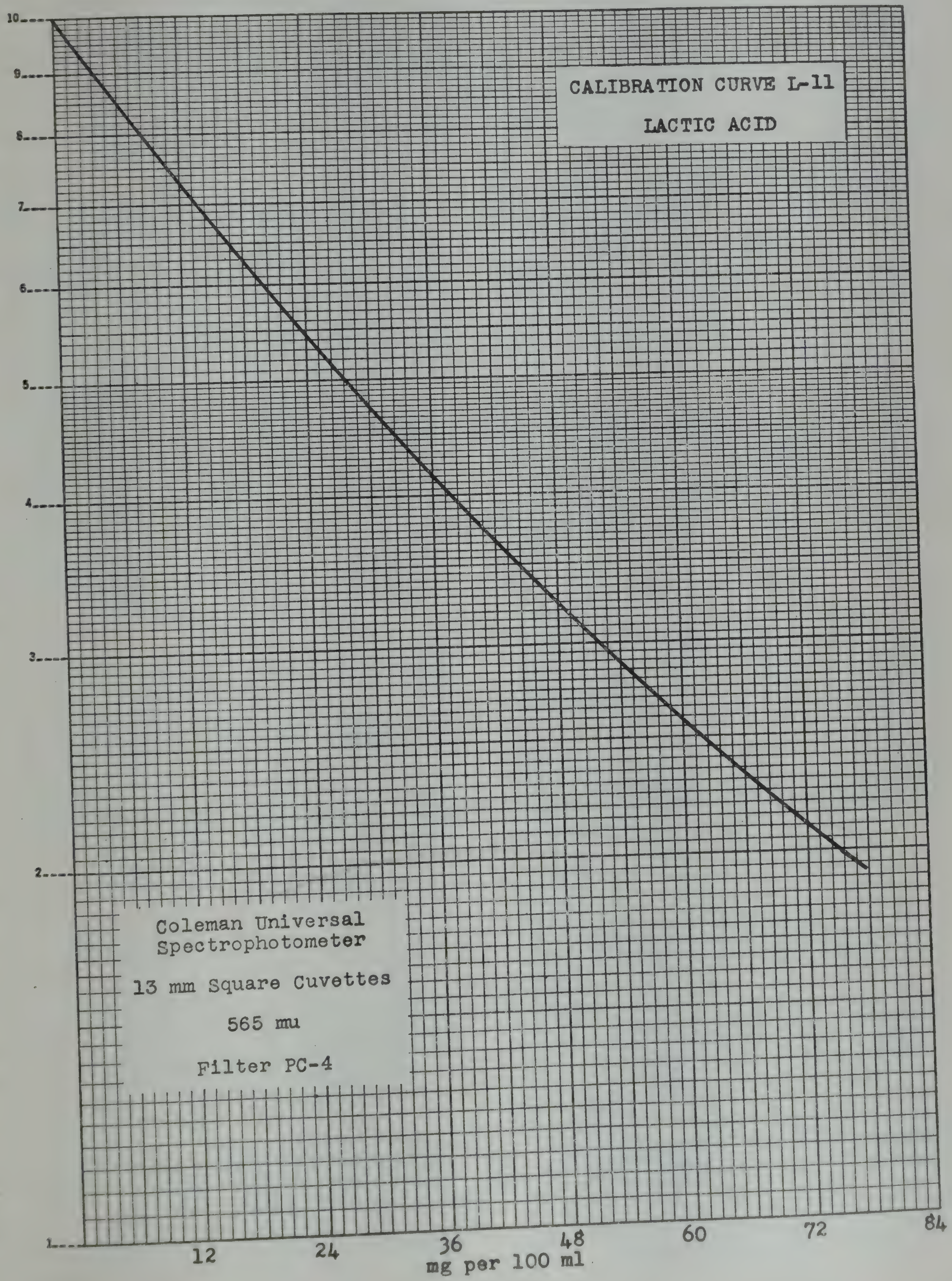

CALIBRATION CURVE L-11

LACTIC ACID

Coleman Universal
Spectrophotometer

13 mm Square Cuvettes

565 mu

Filter PC-4

Meter Reading	0	1	2	3	4	5	6	7	8	9
0										
10										
20										
30										
40										
50										
60										
70										
80										
90										

LEAD

A spectrophotometric adaptation based on the methods and modifications of Ross and Lucas (1933; 1935), Behrens and Taeger (1935), Clifford and Wichmann (1936), Guettel (1939), Kozelka and Kluchesky (1941), Biefeld and Patrick (1942), Sandell (1944) and Fister (1946)

PRINCIPLE

Acidified urine is treated with ammonium oxalate and calcium chloride causing a precipitation of calcium oxalate which completely entrains the lead and leaves interfering phosphates in solution. After centrifugation the residue is digested with perchloric acid and hydrogen peroxide, treated with ammonium citrate (to prevent precipitation of interfering materials) and hydroxylamine hydrochloride (to prevent interference by oxidizing substances) and brought to a pH of 9.5. The lead is then extracted, in the presence of cyanide, as the red lead dithizonate. The red complex is decomposed by shaking with nitric acid and free dithizone is liberated and removed by chloroform, leaving the lead in the acid phase. After adjustment of the pH to 9.5 with ammoniacal cyanide, the lead is re-extracted with an excess of dithizone and the resultant solution (a mixture of red lead dithizonate and green free dithizone) used for spectrophotometric measurement.

DETERMINATION IN URINE

SAMPLE

Aliquot of a 24-hour specimen. Collect in nitric acid-cleaned Pyrex glass flasks or bottles containing 0.5 ml glacial acetic acid (Reagent #3). Any other receptacles used in the collection of the specimen must be nitric acid-cleaned—do not use earthenware utensils. The determination should be carried out as soon as possible after collection—if the specimen must be preserved, add a few drops of toluene. Bismuth is the only element of biological importance that interferes and if its concentration exceeds that of the lead by more than three times, a special procedure is necessary for its elimination.

TEST PROCEDURE

Special Apparatus

Special glass-stoppered centrifuge tubes, Pyrex, containing approximately 105 ml and accurately graduated at 100 ml—these tubes permit the sample to be measured, precipitated and centrifuged and the residue to be digested without transference.

Micro burner, Argand type.

Separatory funnels, Pyrex, 60 ml, pear-shaped.

Filter paper, lead-free; prepared by extracting Whatman #40 paper with 0.005% dithizone solution (Reagent #15) and finally washing with chloroform (Reagent #17) to remove the dithizone.

Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.

Note: All glassware must be Pyrex and all containers should be glass-stoppered. Stopcocks may be lubricated with lead-free white vaseline. Clean glassware should be washed with warm dilute nitric acid, rinsed with ordinary distilled water and then with lead-free distilled water (Reagent #1) and allowed to drain dry—do not use towels. The absence of lead may be confirmed by shaking 10 ml lead-free distilled water, 2 ml 10% potassium cyanide (Reagent #14) and 1 ml 0.005% dithizone solution (Reagent #15) in the apparatus just before use. If no red color develops, lead is absent, and the apparatus may be used after again rinsing with lead-free distilled water. These precautions are absolutely essential if reproducible results are to be obtained.

Reagents

Note: All reagents and chemicals used in their preparation should be tested for the presence of lead; this is especially true of Reagents #4, 5, 9 and 14. Prepare the solutions as outlined; take five times the amount specified in the technic and make just alkaline to thymol blue (Reagent #11) with ammonium hydroxide (Reagent #12); add 5 drops 10% potassium cyanide (Reagent #14) and shake in a separatory funnel with 1 ml 0.005% dithizone solution (Reagent #15)—a cherry red color indicates the presence of lead. Reagents containing more than 1–2 gamma lead in the amounts specified in the technic must be discarded and new solutions prepared from different lots of chemicals.

1. **Water, Distilled, Lead-Free.** Redistil distilled water from an all-glass Pyrex distillation apparatus; it must not contain more than 1 gamma lead per liter. Preserve in resistant-glass bottle. Use for the preparation of all reagents and standards and where indicated in the test procedure.
2. **Bromcresol Green Indicator Solution, 0.04% Aqueous (Clark).** In a 250 ml volumetric flask place 102 mg of the water-soluble sodium salt of bromcresol green ($C_{21}H_{14}Br_4O_5S$—LaMotte); dissolve in lead-free distilled water (1) and dilute to volume. Preserve in resistant-glass bottle.
3. **Acetic Acid, Glacial** (CH_3COOH—Mallinckrodt, A.C.S.).
4. **Ammonium Oxalate, Powder.** Finely pulverize crystalline ammonium oxalate ($(NH_4)_2C_2O_4 \cdot H_2O$—Baker & Adamson, A.C.S.) in a porcelain mortar.
5. **Calcium Chloride, 10% Aqueous.** Dissolve 13.2 gm crystalline calcium chloride ($CaCl_2 \cdot 2H_2O$—Baker & Adamson, A.C.S.) in lead-free distilled water (1) and dilute to 100 ml. Preserve in resistant-glass bottle.
6. **Oxalate Wash Solution (Ross-Lucas).** Dilute 10 ml saturated ammonium oxalate (a) to 1000 ml with lead-free distilled water (1). Preserve in resistant-glass bottle.
   (a) Ammonium oxalate, saturated aqueous; in a 500 ml resistant-glass bottle place 25 gm powdered ammonium oxalate (4); add

approximately 475 ml lead-free distilled water (1) which has been previously heated to 60° C.; shake thoroughly, allow to cool and sediment; use the clear supernatant fluid.

7. **Perchloric Acid, 72%** ($HClO_4$—G. Frederick Smith, Double Vacuum Distilled, Lead Free).
8. **Hydrogen Peroxide, 30%** (H_2O_2—Merck, A.C.S., Superoxol).
9. **Ammonium Citrate Reagent.** Dissolve 50 gm crystalline ammonium citrate ($(NH_4)_2HC_6H_5O_7$—Baker & Adamson, Reagent) in lead-free distilled water (1) and dilute to 100 ml; adjust to pH 8.5–9.0 with concentrated ammonium hydroxide (Reagent #12); shake with several successive portions of 0.005% dithizone solution (Reagent #15) until the dithizone retains its original green color; remove any excess dithizone by shaking with a small portion of chloroform (Reagent #17) and discard the extract. Preserve in resistant-glass bottle.
10. **Hydroxylamine Hydrochloride, 20% Aqueous.** Dissolve 20 gm crystalline hydroxylamine hydrochloride ($NH_2OH \cdot HCl$—Baker & Adamson, Reagent) in approximately 65 ml lead-free distilled water (1) containing a few drops of m-cresol purple indicator solution (a); add concentrated ammonium hydroxide (Reagent #12) to a yellow color; add 1 ml 5% sodium diethyldithiocarbamate (b); allow to stand for 5 minutes; extract with successive portions of chloroform (Reagent #17) until all metal carbamates and excess reagent are removed as evidenced by the absence of a yellow color when a portion of the chloroform extract is treated with a dilute solution of copper sulfate; add re-distilled concentrated hydrochloric acid (HCl—Baker & Adamson, A.C.S.) to a pink color and dilute to 100 ml with lead-free distilled water. Preserve in resistant-glass bottle.
    (a) m-Cresol purple indicator solution, 0.04% aqueous (Clark); in a 250 ml volumetric flask place 104 mg of the water-soluble sodium salt of m-cresol purple ($C_{21}H_{18}O_5S$—LaMotte); dissolve in lead-free distilled water (1) and dilute to volume. Preserve in resistant-glass bottle.
    (b) Sodium diethyldithiocarbamate, 5% aqueous; dissolve 1 gm sodium diethyldithiocarbamate ($(C_2H_5)_2NCSSNa$—Eastman #2596) in lead-free distilled water (1) and dilute to 20 ml.
11. **Thymol Blue Indicator Solution, 0.04% Aqueous (Clark).** In a 250 ml volumetric flask place 103 mg of the water-soluble sodium salt of thymol blue ($C_{27}H_{30}O_5S$—LaMotte); dissolve in lead-free distilled water (1) and dilute to volume. Preserve in resistant-glass bottle.
12. **Ammonium Hydroxide, Concentrated** (NH_4OH—Baker & Adamson, A.C.S.). The hydroxide specified has always been found to be satisfactory without further purification. Other products may require distillation before use, the ammonia gas being collected in cold lead-free distilled water (1) until saturated. Preserve in resistant-glass bottle.
13. **Citric Acid, 10% Aqueous.** Dissolve 10 gm finely crystalline citric acid ($C_6H_8O_7 \cdot H_2O$—Baker & Adamson, A.C.S., Special Low Lead) in lead-free distilled water (1) and dilute to 100 ml. Preserve in resistant-glass bottle.
14. **Potassium Cyanide, 10% Aqueous.** Dissolve 50 gm granular potassium cyanide (KCN—Baker & Adamson, Reagent) in sufficient lead-free distilled water (1) to make 100 ml; transfer to a separatory funnel and extract with successive portions of 0.005% dithizone solution (Reagent #15) until the extract retains a pure green color; repeatedly extract the aqueous phase with chloroform (Reagent #17) until the extract is colorless; dilute with lead-free distilled water to a concentration of 10 gm per 100 ml. Do not filter. Preserve in resistant-glass bottle.
15. **Dithizone, 0.005% in Chloroform.** In a 1000 ml volumetric flask place exactly 50 mg purified diphenylthiocarbazone ($C_6H_5N{:}N \cdot CS \cdot NH \cdot NH \cdot C_6H_5$—Reagent Chemicals, Special); add approximately 500 ml chloroform (Reagent #17); shake until dissolved and dilute to volume with same. Preserve in amber Pyrex tightly sealed glass-stoppered bottle in refrigerator. Protect from direct light. Stability several months.

    Note: Dithizone, other than that specified, may be used provided that when a 0.01% solution in chloroform is shaken with a 1:100 dilution of ammonium hydroxide (12), the color of the chloroform phase becomes only a very pale yellow; if deeper yellow, the product is not satisfactory and must be purified.

16. **Nitric Acid, 1% Aqueous.** Dilute 10 ml concentrated nitric acid (HNO_3—Grasselli, A.C.S.) to 1000 ml with lead-free distilled water (1). Preserve in resistant-glass bottle.
17. **Chloroform** ($CHCl_3$—Baker & Adamson, A.C.S.). Use only chloroform which is received from the manufacturer in glass bottles—do not use bulk chloroform which is usually packed in tins. If necessary, chloroform may be purified by redistilling from a small amount of calcium oxide adding, before distillation is started, 1.5 ml 95% ethyl alcohol for every 100 ml chloroform. Preserve in Pyrex glass-stoppered bottle.
18. **Ammoniacal Potassium Cyanide Solution.** In a 100 ml mixing cylinder place 2 gm potassium cyanide (KCN—Baker & Adamson, Reagent) and approximately 50 ml lead-free distilled water (1); shake until dissolved; add 15 ml concentrated ammonium hydroxide (12); mix and dilute to volume with lead-free distilled water. Preserve in resistant-glass bottle in a cool place.
19. **Dithizone, 0.0005% in Chloroform.** In a 100 ml volumetric flask place exactly 10 ml 0.005% dithizone solution (15) and dilute to volume with

chloroform (17). Preserve in amber Pyrex tightly sealed glass-stoppered bottle in refrigerator. Protect from direct light.

Note: This solution is preferably made up frequently in small amounts. Unless diluted from a freshly prepared 0.005% solution, the concentration of dithizone should be carefully checked against a standard.

20. **Dithizone, 0.001% in Chloroform.** In a 100 ml volumetric flask place exactly 20 ml 0.005% dithizone solution (15) and dilute to volume with chloroform (17). Preserve in amber Pyrex tightly sealed glass-stoppered bottle in refrigerator. Protect from direct light. Note: The note under Reagent #19 applies here also.

Technic

Note: For concentrations of lead in excess of 50 gamma per 100 ml, dilute the acid phase in Step 26 to a definite volume, proceed with an aliquot and correct the values obtained from the calibration table accordingly.

For concentrations less than 5 gamma per 100 ml, greater precision may be attained by the use of a 0.0002% dithizone solution in Step 30; values are obtained from a calibration curve prepared by treating 0–5 gamma standards with a 0.0002% dithizone solution as outlined in the calibration procedure.

1. In a special 100 ml centrifuge tube (sample) place 100 ml thoroughly mixed unfiltered urine.
2. Place several drops of bromcresol green indicator solution (2) on a porcelain spot plate.
3. Add glacial acetic acid (3) to the urine, drop by drop, mixing after each addition, until a drop of the sample changes the green color of the indicator on the spot plate to yellow (pH 4.5).
4. In another special 100 ml centrifuge tube (reagent blank) place 100 ml lead-free distilled water (1) and the same number of drops of glacial acetic acid (3) as used in Step 3.

Note: From this point forward, both sample and reagent blank are treated in an identical manner.

5. Add 0.5 gm powdered ammonium oxalate (4), stopper the tube and shake until dissolved.
6. Add 2 ml 10% calcium chloride (5); mix and allow to stand for 20 minutes, shaking occasionally.
7. Centrifuge the stoppered tube at 2000 rpm for 5 minutes; decant and discard the supernatant fluid.
8. Wash the precipitate with 20 ml oxalate wash solution (6), stir up the precipitate with a glass rod and again centrifuge at 2000 rpm for 5 minutes; decant and discard the supernatant fluid.
9. Invert the tube and drain on filter paper for 3 minutes.
10. To the precipitate in the tube add 2 ml 72% perchloric acid (7) and 2 drops 30% hydrogen peroxide (8) and mix by agitating the tube.
11. Clamp the tube on a support stand under a fume hood and heat gently with a micro burner until the volume is reduced by one-half (not less than 20 minutes).
12. Allow to cool for 1 minute; cautiously add 3 drops 30% hydrogen peroxide (8), allowing the drops to flow down the side of the tube.
13. Continue digestion for 3 minutes; allow to cool to room temperature.
14. Add 5 ml lead-free distilled water (1) and agitate the tube until any precipitate has dissolved.
15. Quantitatively transfer the contents of the tube to a correspondingly marked 60 ml separatory funnel, washing out the tube with about 10 ml lead-free distilled water (1).
16. Add 15 ml ammonium citrate reagent (9) and mix
    1 ml 20% hydroxylamine hydrochloride (10) and mix
    2 drops thymol blue indicator solution (11) and mix.
17. The solution should be blue—if not, add concentrated ammonium hydroxide (12), drop by drop, to the appearance of a blue color. Steps 17–28 inclusive should be carried out without delay.

Note: At this point the solution should be clear; occasionally, however, a slight precipitate may be observed, especially if allowed to stand. To clear, add sufficient 10% citric acid (13), drop by drop, to dissolve the precipitate; if the blue color is discharged, cautiously re-alkalinize with ammonium hydroxide (12).

18. Add 5 ml 10% potassium cyanide (14) and mix.
19. Adjust to pH 9.5 (greenish-blue color), if necessary, by the addition of concentrated ammonium hydroxide (12).
20. Add 5 ml 0.005% dithizone solution (15); stopper the funnel and shake thoroughly for 2 minutes (for very low concentrations of lead use 2.5 ml 0.005% dithizone solution and 2.5 ml chloroform in Steps 20 and 24).

Note: If the approximate concentration of lead in the sample is unknown, the 5 ml dithizone solution should be added in increments of 1 ml, shaking after each addition, until the color deviates from the cherry red of lead dithizonate; note the volume of dithizone added before adding the remainder of the 5 ml—this procedure gives an indication of the concentration of lead and governs the strength of the dithizone to be added later in Step 30 (1 ml 0.005% dithizone is equivalent to approximately 20 gamma of lead).

21. Allow to stand until a clear chloroform layer separates; draw off the chloroform phase into another 60 ml separatory funnel.
22. Repeat Steps 20 and 21 twice—combine the dithizone extracts in the one funnel and discard the yellowish aqueous phase after the final extraction. Note: The last extract must be pure green and show no mixed color.
23. To the combined dithizone extracts add 20 ml lead-free distilled water (1); shake thoroughly for 1 minute; allow to stand until the layers separate and transfer the chloroform phase to another 60 ml separatory funnel.
24. To the funnel containing the aqueous phase add 2 ml 0.005% dithizone solution (15); stopper and shake thoroughly for 1 minute; allow the layers to separate and transfer the dithizone phase to

the funnel containing the original dithizone extract.

25. Add 25 ml 1% nitric acid (16) to the funnel containing the combined dithizone extracts; shake thoroughly for 1 minute; allow the layers to separate and draw off the chloroform phase into another 60 ml separatory funnel.

26. Repeat Step 25, draw off and discard the chloroform phase and combine the two acid phases in the one funnel.

27. Add 2–3 ml chloroform (17) to the combined aqueous acid phases and swirl the solution in the funnel so that the chloroform removes any droplets of dithizone adhering to the sides of the funnel and collects them in one globule; draw off and discard the chloroform, leaving the bore of the stopcock filled with chloroform. Note: If there is a small amount of chloroform floating on the surface of the aqueous phase, allow the funnel to stand unstoppered until it has evaporated.

28. Dry the stem of the funnel with a roll of tissue.

Note: The sample and reagent blank should be carried through consecutively to Step 28 and then simultaneously through the remainder of the procedure.

29. To the acid phase add 10 ml ammoniacal potassium cyanide solution (18), 2 drops thymol blue indicator solution (11) and mix—the color of the solution should be greenish-blue.

30. For low concentrations of lead (0–15 gamma per 100 ml) add 15 ml 0.0005% dithizone solution (19); for higher concentrations (15–50 gamma per 100 ml) add 15 ml 0.001% dithizone solution (20); for concentrations in excess of 50 gamma per 100 ml, see note under technic. Note: The appropriate concentration of dithizone to be used has been previously indicated in Step 20.

31. Stopper, shake thoroughly for 1 minute; allow the layers to separate and draw off a portion of the chloroform phase through a lead-free filter paper into a dry cuvette.

32. Stopper the cuvette with an aluminum foil-covered cork and place in a cool dark place until the companion tube is ready for spectrophotometric measurement—never expose the mixed-color solution to direct light.

33. Within 1 hour, measure the transmittancy of the sample and the reagent blank against distilled water set at 100% transmission at a wave length of **610 mμ** and obtain the values from the appropriate calibration table, using the calibration table prepared from Calibration Curve L-15A for 0.0005% dithizone solutions and that prepared from Calibration Curve L-15B for 0.001% solutions.

34. Calculate the concentration of lead as follows:

Calculation

Value for lead in sample in gamma per 100 ml — Value for lead in reagent blank in gamma per 100 ml = Lead in gamma per 100 ml urine

CALIBRATION PROCEDURE

Special Apparatus

Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.

Note: Do not use flasks or pipettes which have been employed in the preparation of lead standards for the preparation of other reagent solutions or in the test procedure unless they have been scrupulously cleaned as described.

Ignition tubes, Pyrex, heavy wall, 18 × 150 mm.
Micro burner, Argand type.
Separatory funnels, Pyrex, 60 ml, pear-shaped.
Filter paper, lead-free.

Note: See note under Test Procedure.

Reagents

1. **Water, Distilled, Lead-Free.** Same as for Test Procedure.
7. **Perchloric Acid, 72%.** Same as for Test Procedure.
8. **Hydrogen Peroxide, 30%.** Same as for Test Procedure.
9. **Ammonium Citrate Reagent.** Same as for Test Procedure.
10. **Hydroxylamine Hydrochloride, 20% Aqueous.** Same as for Test Procedure.
11. **Thymol Blue Indicator Solution, 0.04% Aqueous (Clark).** Same as for Test Procedure.
12. **Ammonium Hydroxide, Concentrated.** Same as for Test Procedure.
13. **Citric Acid, 10% Aqueous.** Same as for Test Procedure.
14. **Potassium Cyanide, 10% Aqueous.** Same as for Test Procedure.
15. **Dithizone, 0.005% in Chloroform.** Same as for Test Procedure.
16. **Nitric Acid, 1% Aqueous.** Same as for Test Procedure.
17. **Chloroform.** Same as for Test Procedure.
18. **Ammoniacal Potassium Cyanide Solution.** Same as for Test Procedure.
19. **Dithizone, 0.0005% in Chloroform.** Same as for Test Procedure.
20. **Dithizone, 0.001% in Chloroform.** Same as for Test Procedure.
21. **Lead Standards**
    (a) Lead stock standard (1 ml contains 0.1 mg lead). In a 1000 ml volumetric flask place 159.9 mg dry crystalline lead nitrate ($Pb(NO_3)_2$—Baker & Adamson, A.C.S.); add approximately 250 ml lead-free distilled water (1) and 10 ml concentrated nitric

acid (HNO_3—Grasselli, A.C.S.); shake until dissolved and dilute to volume with lead-free distilled water. Preserve in resistant-glass bottle.

(b) Dilute standard (1 ml contains 0.01 mg lead). In a 100 ml volumetric flask place 10 ml stock standard (21a) and dilute to volume with lead-free distilled water (1). Preserve in resistant-glass bottle. Prepare just before use.

(c) Calibration standards for the 0–15 gamma range. In a series of six 18 × 150 mm Pyrex ignition tubes place respectively 0, 0.1, 0.25, 0.5, 1.0 and 1.5 ml of the dilute standard (21b); add 5.0, 4.9, 4.75, 4.5, 4.0 and 3.5 ml lead-free distilled water (1) respectively, bringing the volume in each tube to 5 ml. These standards represent concentrations of lead equivalent to 0, 1, 2.5, 5, 10 and 15 gamma per 100 ml sample. Prepare just before use and proceed immediately.

(d) Calibration standards for the 0–50 gamma range. In another series of six 18 × 150 mm Pyrex ignition tubes place respectively 0, 1, 2, 3, 4 and 5 ml of the dilute standard (21b); add 5, 4, 3, 2, 1 and 0 ml lead-free distilled water (1) respectively, bringing the volume in each tube to 5 ml. These standards represent concentrations of lead equivalent to 0, 10, 20, 30, 40 and 50 gamma per 100 ml sample. Prepare just before use and proceed immediately.

Technic

Note: When preparing the original calibration curves and each time an analysis is made, note the transmission of the dithizone solutions against distilled water set at 100% transmission at a wave length of **610** mμ. If the readings deviate from those originally recorded, the dithizone solutions have deteriorated and should be replaced.

1. To each calibration standard (21c and 21d) add
   2 ml 72% perchloric acid (7)
   2 drops 30% hydrogen peroxide (8) and mix.
2. Proceed exactly as outlined in the Test Procedure, Steps 11–32 inclusive, using 0.0005% dithizone solution in Step 30 for the 0–15 gamma range and 0.001% dithizone solution in Step 30 for the 0–50 gamma range.
3. Set distilled water at 100% transmission at a wave length of **610** mμ and record the transmittancy of the zero concentration and standard tubes in both series.
4. Correction must be made for the color of the zero concentration standards. Divide the transmittancy reading of each lead standard in the 0–15 gamma range by the transmittancy reading of the zero concentration standard for this range and subtract these values from the transmittancy readings of the respective standards, or expressed as a formula—

$$\text{Transmittancy \% of standard} - \left(\frac{\text{Transmittancy \% of standard}}{\text{Transmittancy \% of O concentration}}\right) = \text{Corrected transmittancy \% of standard}$$

   In the same manner, correct for the color of the zero concentration standard in the 0–50 gamma range.
5. Plot the corrected values on semi-log graph paper—the points fall on a straight line which slopes upward and passes through the transmission axis at approximately 46% for the 0–15 gamma range (Calibration Curve L-15A) and at approximately 20% for the 0–50 gamma range (Calibration Curve L-15B); the transmittancy of the 15 gamma standard (with 0.0005% dithizone) should be approximately 67% and that of the 50 gamma standard (with 0.001% dithizone) should be approximately 70%.
6. Prepare calibration tables from the T-C values obtained from the plotted curves.

PROCEDURE FOR USE WITH LINEAR-SCALE PHOTOELECTRIC COLORIMETERS (SHEARD-SANFORD TYPE)

TEST PROCEDURE

Exactly as outlined; set distilled water at 100 and use an orange filter with maximum transmission at **610** mμ.

CALIBRATION PROCEDURE

Exactly as outlined—use an orange filter with maximum transmission at **610** mμ; plot the corrected values on semi-log graph paper—the points fall on a straight line which passes through the lower end of the transmission axis and slopes upward.

PROCEDURE FOR USE WITH LOGARITHMIC-SCALE PHOTOELECTRIC COLORIMETERS (KLETT-SUMMERSON TYPE)

TEST PROCEDURE

Exactly as outlined; set distilled water at 0 and use an orange filter with maximum transmission at **610** mμ; subtract the reading of the sample from the reading of the reagent blank and obtain the concentration of lead, represented by the difference in density, from the calibration table.

CALIBRATION PROCEDURE

Exactly as outlined—use an orange filter with maximum transmission at **610** mμ. Correction must be made for the color of the zero concentration standards as follows:

(a) Subtract the reading of the first lead standard in the series from the reading of the zero concentration standard and divide the difference by the concentration of the standard, or expressed as a formula—

$$\frac{\begin{array}{c}\text{Reading of 0}\\ \text{concentration}\\ \text{standard}\end{array} - \begin{array}{c}\text{Reading}\\ \text{of}\\ \text{standard}\end{array}}{\text{Concentration of standard}} = \text{C value}$$

(b) In the same manner, subtract the reading of the second lead standard in the series from the reading of the zero concentration standard and divide the difference by the concentration of the standard.

(c) Calculate the C values for the remaining standards in the series in the same manner. If the calibration procedure has been carefully carried out, all C values should be identical; if not, take the average of all the C values—this value represents the optical density change produced by 1 gamma of lead.

(d) Multiply the concentration of each standard by the averaged C value and plot the results on linear graph paper, assigning the concentration values of the standards to the horizontal axis and the corresponding optical density differences to the vertical axis—the points fall on a straight line passing through the origin.

(e) The curve for the second series of standards is plotted in the same manner.

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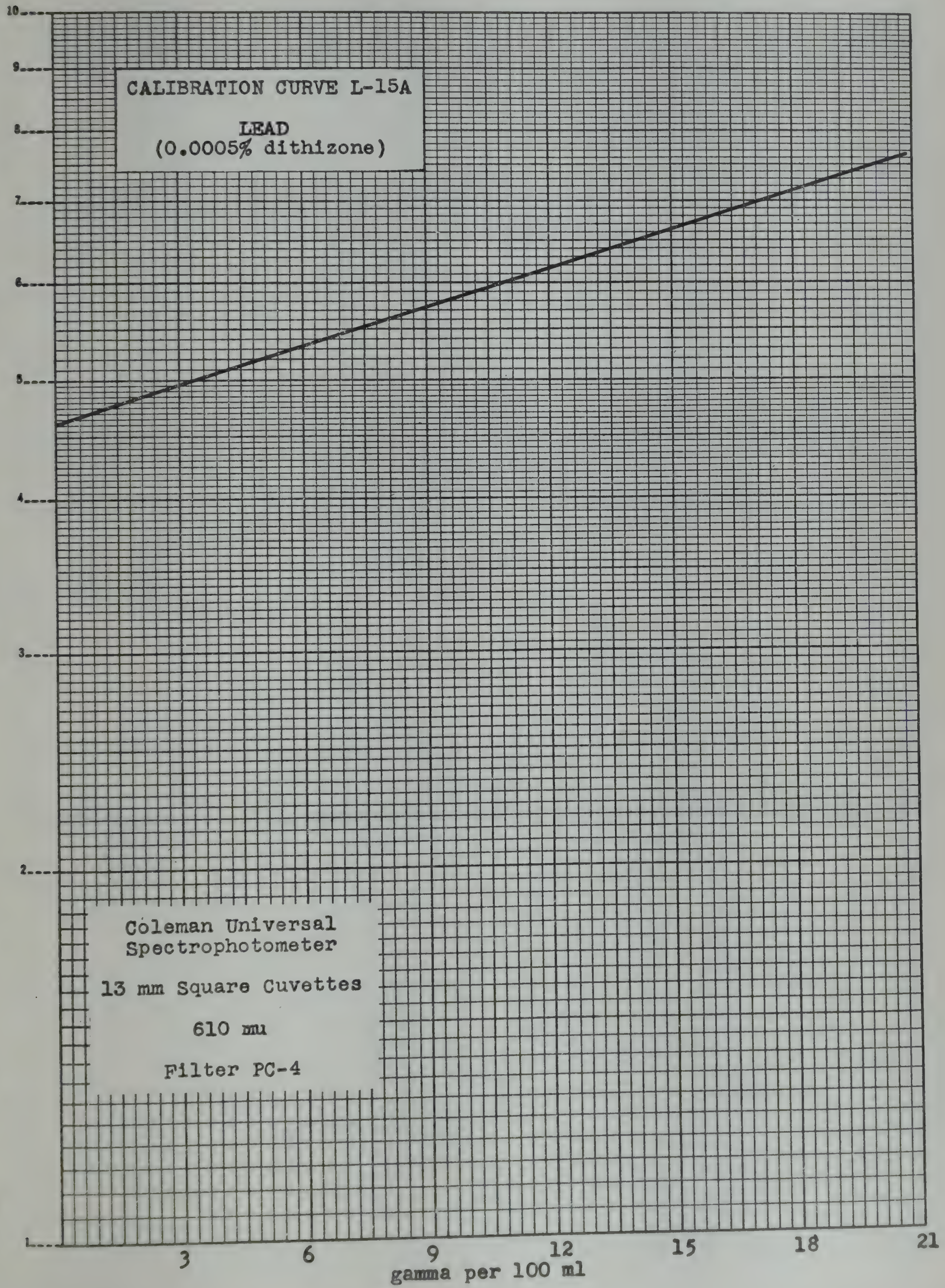
NOTES



CALIBRATION CURVE L-15A

LEAD
(0.0005% dithizone)

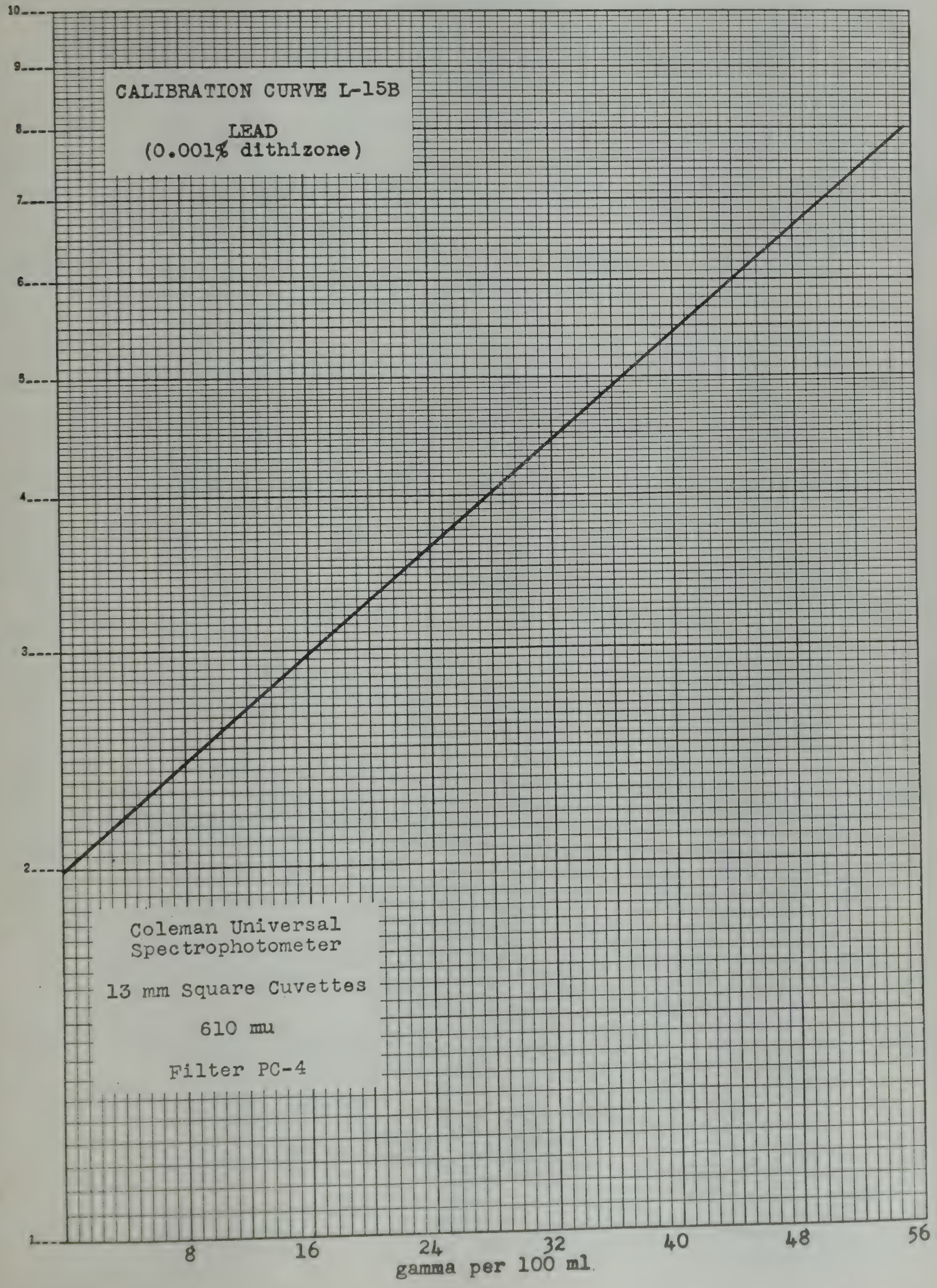
Coleman Universal
Spectrophotometer

13 mm Square Cuvettes

610 mμ

Filter PC-4

Meter Reading	0	1	2	3	4	5	6	7	8	9
0										
10										
20										
30										
40										
50										
60										
70										
80										
90										


CALIBRATION CURVE L-15B
LEAD
(0.001% dithizone)
Coleman Universal
Spectrophotometer
13 mm Square Cuvettes
610 mu
Filter PC-4
10
9
8
7
6
5
4
3
2
1
8
16
24
32
40
48
56
gamma per 100 ml

Meter Reading	0	1	2	3	4	5	6	7	8	9
0										
10										
20										
30										
40										
50										
60										
70										
80										
90										

MAGNESIUM

A spectrophotometric adaptation based on the methods and modifications of Kolthoff (1927), Hirschfelder, Serles and Haury (1934), Haury (1938) and Kunkel, Pearson and Schweigert (1947)

PRINCIPLE

Magnesium, in a protein-free filtrate, the pH of which is carefully adjusted, is precipitated as the hydroxide in the presence of titan yellow. The deep red color lake formed (Kolthoff reaction) is adsorbed on the precipitated hydroxide and produces a colloidal suspension, stabilized by means of starch. Hydroxylamine is used to prevent fading of the color and sucrose is added to minimize the effect of any excess calcium.

DETERMINATION IN PLASMA OR SERUM

SAMPLE

Plasma or serum from blood withdrawn during post-absorptive state. Anticoagulant for plasma—potassium or sodium oxalate—ammonium oxalate must not be used. Analyses preferably made on serum.

TEST PROCEDURE

Special Apparatus

Ostwald-Folin pipettes accurately calibrated "to contain" 2 ml.

Capillary droppers delivering 40 drops per ml.

Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.

Reagents

Note: The distilled water used in the preparation of the reagents and standards and where indicated in the test procedure must be Mg- and Ca-free. One ml distilled water must show no pink color upon the addition of a drop of titan yellow and a drop of sodium hydroxide; no precipitate or turbidity must develop upon treatment with oxalic acid.

1. **Sulfuric Acid, Volumetric 2/3 N.** Slowly add 20 ml concentrated sulfuric acid (H_2SO_4—Grasselli, A.C.S.) to 1000 ml distilled water and mix well; titrate against standard 1 N alkali using phenolphthalein as indicator; adjust accordingly and re-titrate—20 ml of 2/3 N acid require 13.3 ml of 1 N alkali.
2. **Sodium Tungstate, 10% Aqueous.** Dissolve 10 gm sodium tungstate ($Na_2WO_4 \cdot 2H_2O$—Mallinckrodt, Folin Special) in distilled water and dilute to 100 ml. The solution must be neutral or slightly alkaline to phenolphthalein, not more than 0.4 ml being required to neutralize 10 ml 0.1 N hydrochloric acid. Preserve in resistant-glass bottle.
3. **Methyl Red Indicator Solution, 0.01% Alcoholic.** Dissolve 50 mg methyl red ($C_{15}H_{15}O_2N_3$—National Aniline) in 50 ml absolute methyl alcohol (CH_3OH—Merck, A.C.S.).
4. **Sodium Hydroxide, Volumetric 0.1 N.** Prepare by diluting slightly more than the required amount of concentrated carbonate-free sodium hydroxide solution (see Appendix) and standardize against 0.1 N acid using phenolphthalein as indicator. Preserve in ceresin-lined bottle.

Note: When prepared in the manner indicated from carbonate-free sodium hydroxide, Reagent #4 and Reagent #9 are both Mg- and Ca-free.

5. **Sucrose, 5% Aqueous.** Dissolve 5 gm sucrose ($C_{12}H_{22}O_{11}$—Mallinckrodt, AR) in distilled water and dilute to 100 ml. Preserve with a few drops of chloroform.
6. **Hydroxylamine Hydrochloride, 2% Aqueous.** Dissolve 2 gm hydroxylamine hydrochloride ($NH_2OH \cdot HCl$—J. T. Baker, CP Analyzed) in distilled water and dilute to 100 ml. Preserve in a brown glass bottle.
7. **Starch Solution, 1.5% Aqueous.** Triturate 1.5 gm powdered soluble starch ($C_6H_{10}O_5$—Merck, According to Lintner) with 5 ml distilled water and add to 75 ml boiling distilled water; boil for one minute; cool to room temperature and dilute to 100 ml. Prepare fresh daily and filter before use. Note: One ml of the starch solution should be tested for the presence of magnesium and calcium as described in the note under reagents.
8. **Titan Yellow, 0.075% Aqueous.** Dissolve 75 mg titan yellow ($C_{28}H_{19}O_6N_5S_4Na_2$—C.I. #813—Reagent Chemicals, Special) in distilled water and dilute to 100 ml; filter. Preserve in a brown glass bottle in refrigerator. Keep away from direct light at all times. Stability approximately 10 days. Note: Clayton yellow or titan yellow may be used interchangeably provided that the calibration procedure is carried out with the appropriate dye.
9. **Sodium Hydroxide, Volumetric 1.5 N.** Prepare by diluting slightly more than the required amount of concentrated carbonate-free sodium hydroxide solution (see Appendix); titrate against standard 1 N acid using phenolphthalein as indicator; adjust accordingly and re-titrate—30 ml of 1 N acid require 20 ml of 1.5 N alkali. Preserve in ceresin-lined bottle.

Technic

1. In a 15 ml centrifuge tube place
   4 ml distilled water.
2. Add 2 ml sample from an Ostwald-Folin pipette, thoroughly rinsing out the pipette by drawing in and expelling some of the mixture several times.

3. Add 2 ml 2/3 N sulfuric acid (1) and mix by gently tapping the tube until the mixture is uniformly black-brown.
4. Add 2 ml 10% sodium tungstate (2), drop by drop, while constantly but gently, agitating the tube.
5. Shake the tube vigorously until the mixture is chocolate colored and foam-free.
6. Centrifuge at 1500 rpm for 3 minutes.
7. In a 10 ml volumetric flask (sample) place
   3 ml clear supernatant centrifugate.
8. In another 10 ml volumetric flask (reagent blank) place
   3 ml distilled water.
9. To each flask add 1 drop methyl red indicator solution (3) and carefully add 0.1 N sodium hydroxide (4), drop by drop, by means of a capillary-tipped dropper, to the yellow color of the indicator—avoid an excess.
10. Add 1 ml 5% sucrose (5) and mix.
11. Add 1 ml 2% hydroxylamine hydrochloride (6) and mix.
12. Add 1.5 ml 1.5% starch solution (7) and mix.
13. Add 1 ml 0.075% titan yellow (8) and mix.
14. Add 2 ml 1.5 N sodium hydroxide (9) and mix.
15. Immediately dilute to volume with distilled water and mix thoroughly.
16. Place in water bath at 25° C. for 10 minutes.
17. Measure the transmittancy of the sample against the reagent blank set at 100% transmission at a wave length of **540 mμ** and obtain the concentration of magnesium from the calibration table.

CALIBRATION PROCEDURE

Special Apparatus

Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.

Reagents

3. **Methyl Red Indicator Solution, 0.01% Alcoholic.** Same as for Test Procedure.
4. **Sodium Hydroxide, Volumetric 0.1 N.** Same as for Test Procedure.
5. **Sucrose, 5% Aqueous.** Same as for Test Procedure.
6. **Hydroxylamine Hydrochloride, 2% Aqueous.** Same as for Test Procedure.
7. **Starch Solution, 1.5% Aqueous.** Same as for Test Procedure.
8. **Titan Yellow, 0.075% Aqueous.** Same as for Test Procedure.
9. **Sodium Hydroxide, Volumetric 1.5 N.** Same as for Test Procedure.
10. **Magnesium Standards**
    (a) Magnesium stock standard (1 ml contains 1 mg magnesium). In a 100 ml volumetric flask place 100 mg powdered metallic magnesium (Mg—Mallinckrodt, Powder); add 5 ml distilled water and 2.5 ml concentrated hydrochloric acid (HCl—Baker & Adamson, A.C.S.); shake until dissolved and dilute to volume with distilled water. Preserve with 2 ml chloroform.
    (b) Dilute standard (1 ml contains 0.03 mg magnesium). In a 100 ml volumetric flask place 3 ml stock standard (10a) and dilute to volume with distilled water. Prepare day of use.
    (c) Calibration standards. In a series of five accurately calibrated 10 ml volumetric flasks place respectively 0, 0.5, 1, 1.5 and 2 ml of the dilute standard (10b); add 3, 2.5, 2, 1.5 and 1 ml distilled water respectively, bringing the volume in each flask to 3 ml. These standards represent concentrations of magnesium equivalent to 0, 2.5, 5, 7.5 and 10 mg per 100 ml sample. Proceed immediately as follows.

Technic

1. Treat each calibration standard (10c) exactly as outlined in the Test Procedure, Steps 9–16 inclusive.
2. Set the zero concentration standard at 100% transmission at a wave length of **540 mμ** and record the transmittancy of the 2.5, 5, 7.5 and 10 mg per cent standards. The transmittancy of the 10 mg standard should be approximately 35%.
3. Plot the observed values on semi-log graph paper—the points fall on a practically straight line which passes through the origin (Calibration Curve M-10).
4. Prepare a calibration table from the T-C values obtained from the plotted curve.

Method M-10b

DETERMINATION IN WHOLE BLOOD

SAMPLE

See remarks under plasma or serum.

TEST PROCEDURE

Special Apparatus

Ostwald-Folin pipettes accurately calibrated "to contain" 1 ml.

Capillary droppers delivering 40 drops per ml.

Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.

Reagents

1. **Sulfuric Acid, Volumetric 2/3 N.** Same as for plasma or serum.

2. **Sodium Tungstate, 10% aqueous.** Same as for plasma or serum.
3. **Methyl Red Indicator Solution, 0.01% Alcoholic.** Same as for plasma or serum.
4. **Sodium Hydroxide, Volumetric 0.1 N.** Same as for plasma or serum.
5. **Sucrose, 5% Aqueous.** Same as for plasma or serum.
6. **Hydroxylamine Hydrochloride, 2% Aqueous.** Same as for plasma or serum.
7. **Starch Solution, 1.5% Aqueous.** Same as for plasma or serum.
8. **Titan Yellow, 0.075% Aqueous.** Same as for plasma or serum.
9. **Sodium Hydroxide, Volumetric 1.5 N.** Same as for plasma or serum.

Technic

1. In a 15 ml centrifuge tube place
   7 ml distilled water.
2. Add 1 ml oxalated blood from an Ostwald-Folin pipette, thoroughly rinsing out the pipette by drawing in and expelling some of the mixture several times.
3. Add 1 ml 2/3 N sulfuric acid (1) and mix by gently tapping the tube until the mixture is uniformly black-brown.
4. Add 1 ml 10% sodium tungstate (2), drop by drop, while constantly but gently, agitating the tube.
5. Shake the tube vigorously until the mixture is chocolate colored and foam-free.
6. Proceed exactly as outlined in the Test Procedure for plasma or serum, Steps 6–16 inclusive.
7. Measure the transmittancy of the sample against the reagent blank set at 100% transmission at a wave length of 540 mμ and multiply the value from the calibration table for plasma or serum by 2 to obtain the concentration of magnesium in mg per 100 ml blood.

Method M-10c

DETERMINATION IN URINE

SAMPLE

Aliquot of a 24-hour specimen. Keep cold during collection.

TEST PROCEDURE

Special Apparatus

Capillary droppers delivering 40 drops per ml.
Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.

Reagents

1. **Sulfuric Acid, Volumetric 2/3 N.** Same as for plasma or serum.
2. **Sodium Tungstate, 10% Aqueous.** Same as for plasma or serum.
3. **Methyl Red Indicator Solution, 0.01% Alcoholic.** Same as for plasma or serum.
4. **Sodium Hydroxide, Volumetric 0.1 N.** Same as for plasma or serum.
5. **Sucrose, 5% Aqueous.** Same as for plasma or serum.
6. **Hydroxylamine Hydrochloride, 2% Aqueous.** Same as for plasma or serum.
7. **Starch Solution, 1.5% Aqueous.** Same as for plasma or serum.
8. **Titan Yellow, 0.075% Aqueous.** Same as for plasma or serum.
9. **Sodium Hydroxide, Volumetric 1.5 N.** Same as for plasma or serum.
11. **Hydrochloric Acid, Concentrated** (HCl—Baker & Adamson, A.C.S.).

Technic

Note: For concentrations of magnesium less than 10 mg per 100 ml, dilute a larger volume of urine to 10 ml in Step 3 and for concentrations in excess of 40 mg per 100 ml, dilute a smaller volume of urine to 10 ml in Step 3; correct the values obtained from the calibration table for plasma or serum accordingly.

1. Place a 25 ml aliquot of the thoroughly mixed 24-hour specimen in a small Erlenmeyer flask and add 1–2 drops concentrated hydrochloric acid (11) to dissolve any precipitated phosphate.
2. Filter a small portion of the acidified urine.
3. Dilute 2.5 ml filtered urine to 10 ml with distilled water.
4. In a 15 ml centrifuge tube place
   4 ml distilled water
   2 ml diluted urine
   2 ml 2/3 N sulfuric acid (1)
   2 ml 10% sodium tungstate (2) and shake vigorously.
5. Proceed exactly as outlined in the Test Procedure for plasma or serum, Steps 6–16 inclusive.
6. Measure the transmittancy of the sample against the reagent blank set at 100% transmission at a wave length of 540 mμ and multiply the value from the calibration table for plasma or serum by 4 to obtain the concentration of magnesium in mg per 100 ml urine.

Calculation

$$\frac{\text{Volume of 24-hour specimen}}{100} \times \text{Magnesium in mg per 100 ml} = \text{Mg magnesium excreted in 24 hours}$$

PROCEDURE FOR USE WITH LINEAR-SCALE PHOTOELECTRIC COLORIMETERS (SHEARD-SANFORD TYPE)

TEST PROCEDURE

Exactly as outlined; set the reagent blank at 100 and use a green filter.

CALIBRATION PROCEDURE

Exactly as outlined—use a green filter; plot the observed readings on semi-log graph paper—the points fall on an extremely slight curve passing through the origin.

PROCEDURE FOR USE WITH LOGARITHMIC-SCALE PHOTOELECTRIC COLORIMETERS (KLETT-SUMMERSON TYPE)

TEST PROCEDURE

Exactly as outlined; set the reagent blank at 0 and use a green filter.

CALIBRATION PROCEDURE

Exactly as outlined—use a green filter; plot the observed readings on linear graph paper—the points fall on an extremely slight curve passing through the origin.

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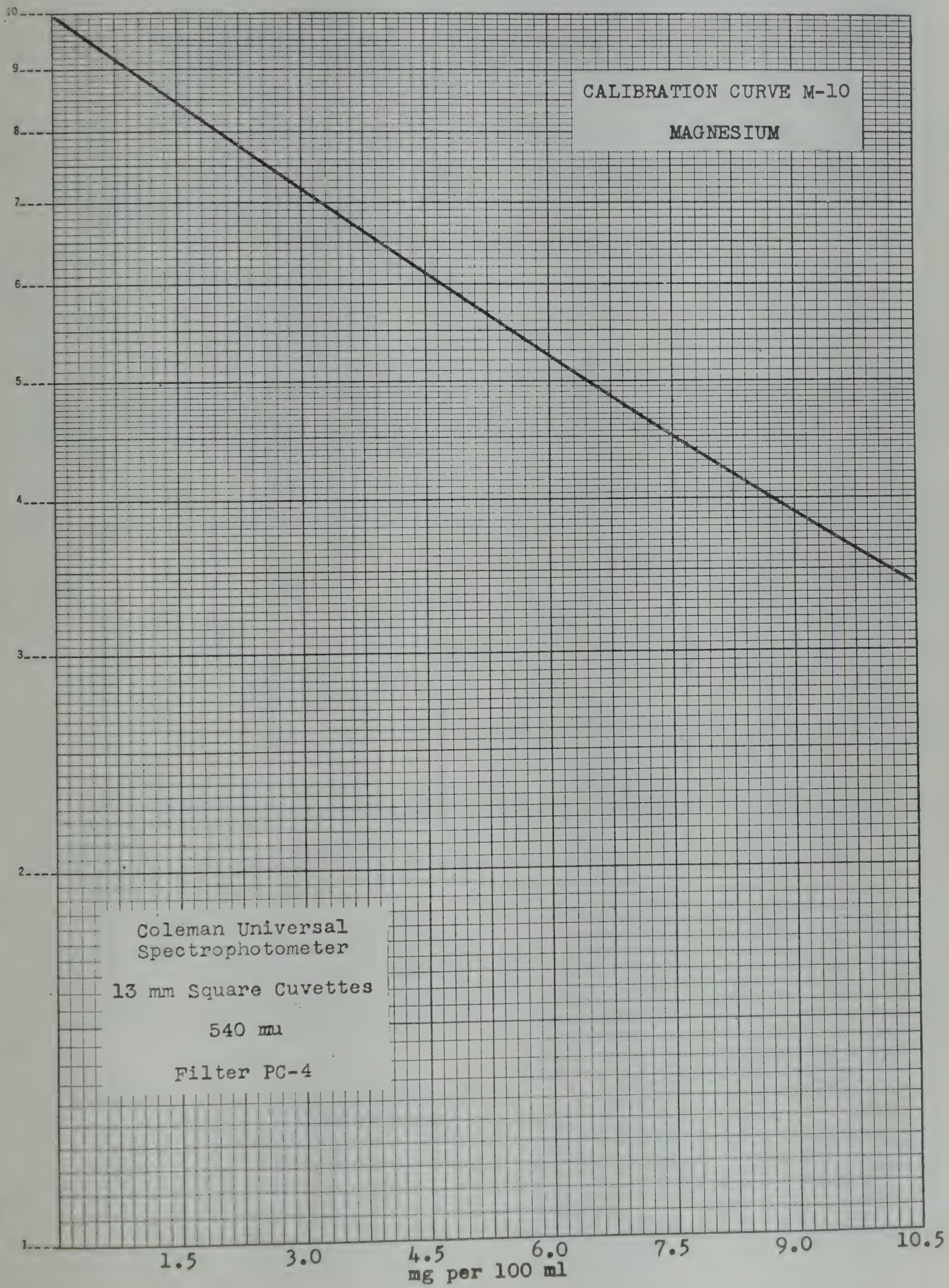
NOTES


CALIBRATION CURVE M-10
MAGNESIUM
Coleman Universal Spectrophotometer
13 mm Square Cuvettes
540 mu
Filter PC-4
10
9
8
7
6
5
4
3
2
1
1.5
3.0
4.5
6.0
7.5
9.0
10.5
mg per 100 ml

Meter Reading	0	1	2	3	4	5	6	7	8	9
0										
10										
20										
30										
40										
50										
60										
70										
80										
90										

MERCURY

A spectrophotometric adaptation based on the methods and modifications of Fischer and Leopoldi (1935), Laug and Nelson (1942) and Maren (1943)

PRINCIPLE

The organic matter in the sample is oxidized by means of a mixture of nitric and sulfuric acids. The digest is then treated with hydroxylamine hydrochloride to destroy excess nitric acid and prevent interference by other oxidizing agents. The pH is adjusted to 1.0, shaken with diphenylthiocarbazone (dithizone) and the mercury extracted as the orange-yellow dithizonate. This is a mixed-color method and, under the conditions outlined, the excess green dithizone need not be destroyed or separated from the yellow mercury dithizonate, thus offering the analyst the choice of measuring the transmittancy of either the excess dithizone (maximum absorption in the red) or the amount of mercury dithizonate formed (maximum absorption in the blue). The latter procedure is outlined in the following method.

DETERMINATION IN WHOLE BLOOD AND URINE

SAMPLE

Oxalated whole blood or aliquot of a 24-hour urine specimen. Samples should be collected in Pyrex glass-stoppered bottles taking precautions to avoid contamination by mercury. Rubber stoppers must never be used. Gold, platinum and palladium interfere.

TEST PROCEDURE

Special Apparatus

Digestion apparatus consisting of a 250 ml round-bottom Pyrex boiling flask with two necks, each with ground glass joint; an air condenser 100 cm long with ground glass joint is fitted to the center neck and a 50 ml dropping funnel with ground glass joint to the other neck (Fig. M-15).

Separatory funnels, Pyrex, 125 ml, pear-shaped.

Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.

Note: All glassware must be washed with warm 25% nitric acid, thoroughly rinsed with distilled water and dried before use.

Reagents

1. **Nitric-Sulfuric Acid Digestion Mixture (Maren).** Carefully mix 130 ml concentrated nitric acid (HNO_3—Grasselli, A.C.S.) with 110 ml concentrated sulfuric acid (H_2SO_4—Grasselli, A.C.S.). Preserve in Pyrex glass-stoppered bottle.
2. **Hydrochloric Acid, Volumetric 0.25 N.** Add 22 ml concentrated hydrochloric acid (HCl—Baker & Adamson, A.C.S.) to 1000 ml distilled water and mix; titrate against standard 0.1 N alkali using phenolphthalein as indicator; adjust accordingly and re-titrate—10 ml of 0.25 N acid require 25 ml of 0.1 N alkali. Preserve in Pyrex glass-stoppered bottle. Note: If a blank analysis shows the presence of mercury, the concentrated acid must be redistilled from an all-glass Pyrex distillation apparatus.

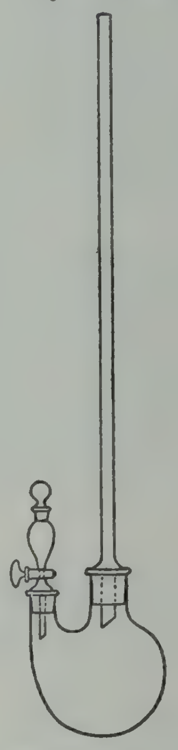


Fig. M-15.

3. **Hydroxylamine Hydrochloride, 20% Aqueous.** Dissolve 20 gm hydroxylamine hydrochloride ($NH_2OH \cdot HCl$—J. T. Baker, CP Analyzed) in distilled water and dilute to 100 ml; transfer to a 250 ml separatory funnel and add 2 ml 0.05% dithizone (Reagent #4a) and 8 ml chloroform; shake vigorously for one minute, allow the phases to separate, draw off and discard the chloroform layer; repeat the washing with dithizone and chloroform in the same manner twice more. Preserve in a brown Pyrex glass-stoppered bottle.
4. **Diphenylthiocarbazone, 0.001% in Chloroform.** Dilute 2 ml 0.05% diphenylthiocarbazone (a) to 100 ml with anhydrous, acid-washed, redistilled chloroform ($CHCl_3$—Reagent Chemicals, Special, according to Biddle and Clifford). Prepare fresh daily and keep in a brown Pyrex glass-stoppered bottle in refrigerator. No dithizone solution should ever be exposed to direct sunlight.
    (a) Diphenylthiocarbazone, 0.05% in chloroform; in a 100 ml volumetric flask place 50 mg recrystallized diphenylthiocarbazone ($C_{13}H_{12}N_4S$—Reagent Chemicals, Special); add about 50 ml anhydrous, acid-washed, redistilled chloroform ($CHCl_3$—Reagent Chemicals, Special, according to Biddle and Clifford); shake until dissolved and dilute to volume with same. Preserve in refrigerator in a brown Pyrex glass-stoppered bottle. Stability approximately one month.

Note: On prolonged standing, crystalline dithizone undergoes oxidation with the formation of a yellow oxidation product. The dithizone should be checked at intervals for purity as follows: shake 5 ml 0.05% dithizone with 10 ml mercury-free 1:100 ammonium hydroxide and allow the phases to separate—the appearance of a light yellow to brown color in the chloroform layer indicates the necessity for further purification (for method see any standard text on organic reagents).

Technic

Note: For concentrations of mercury in excess of 0.4 mg per 100 ml, dilute the digested sample to a definite volume before proceeding with Step 8, so that a 25 ml aliquot contains less than 25 y mercury; for concentrations less than 0.1 mg per 100 ml, use a larger sample and a correspondingly increased amount of digestion mixture in Step 4. In either case, correct the values obtained from the calibration table accordingly.

1. In a 250 ml double-neck Pyrex boiling flask place 5 ml sample.
2. Insert an air condenser into the center opening and a 50 ml dropping funnel into the other.
3. Clamp the apparatus in an upright position in a fume hood.
4. Place 20 ml nitric-sulfuric acid digestion mixture (1) in the dropping funnel and allow to slowly drop into the flask, shutting off the flow before the funnel is entirely empty—digestion begins spontaneously.
5. Allow to stand for 10 minutes and then apply gentle heat until all foaming has subsided—avoid a vigorous reaction and excessive foaming.
6. Increase heat gradually until full flame of burner is used and continue heating for 1 hour after most of the sample has been brought into solution—the brown fumes of nitric acid cease to be evolved and white SO_3 fumes appear.

Note: Occasional charring may occur—in such cases, remove flame, allow to cool for several minutes, slowly add 5–10 ml concentrated nitric acid from the dropping funnel and resume heating—repeat if necessary.

7. Allow the digest to cool to room temperature and then remove the funnel and condenser.
8. Quantitatively transfer the digest to a 125 ml separatory funnel, washing out the flask with successive 5 ml portions of 0.25 N hydrochloric acid (2) until a total of 25 ml has been used, transferring the washings to the separatory funnel.
9. Add 2 ml 20% hydroxylamine hydrochloride (3) and mix.

Note: Mercury dithizonate is photosensitive—carry out Steps 10–14 inclusive as rapidly as possible and away from all direct light.

10. Add 10 ml 0.001% dithizone (4) and shake vigorously for 1 minute.
11. Allow to stand in the dark just long enough for the phases to separate.
12. Draw off the chloroform layer, transferring to a cuvette (sample) through a small funnel containing a small piece of cotton.
13. In another cuvette (reagent blank) place 10 ml 0.001% dithizone (4).
14. Immediately measure the transmittancy of the sample against the reagent blank set at 100% transmission at a wave length of 490 mμ and obtain the concentration of mercury from the calibration table.

Note: The color of the reagent blank is always green—that of the sample may vary from green to yellow—if a deep orange color is obtained (mercury in excess of 25 y per sample) repeat the analysis (see note under Technic).

CALIBRATION PROCEDURE

Note: The calibration curve should be checked frequently, especially with every new lot of dithizone.

Special Apparatus

Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.

Separatory funnels, Pyrex, 125 ml, pear-shaped.

Reagents

2. **Hydrochloric Acid, Volumetric 0.25 N.** Same as for Test Procedure.
4. **Diphenylthiocarbazone, 0.001% in Chloroform.** Same as for Test Procedure.
5. **Mercury Standards**
   (a) Mercury stock standard (1 ml contains 1 mg mercury). In a 100 ml volumetric flask place 135.4 mg crystalline mercuric chloride ($HgCl_2$—Merck, A.C.S.); add about 50 ml 0.25 N hydrochloric acid (2); shake until dissolved and dilute to volume with same. Preserve in Pyrex glass-stoppered bottle.
   (b) Dilute standard (1 ml contains 0.01 mg mercury). In a 100 ml volumetric flask place 1 ml stock standard (5a) and dilute to volume with 0.25 N hydrochloric acid (2). Prepare just before use.
   (c) Calibration standards. In a series of five accurately calibrated 100 ml volumetric flasks place respectively 0, 2, 4, 6 and 8 ml of the dilute standard (5b) and dilute to volume with 0.25 N hydrochloric acid (2). These standards represent concentrations of mercury equivalent to 0, 0.1, 0.2, 0.3 and 0.4 mg per 100 ml sample. Prepare just before use.

Technic

1. Transfer 25 ml of each calibration standard (5c) to a correspondingly marked 125 ml separatory funnel.
2. Proceed exactly as outlined in the Test Procedure, Steps 10–12 inclusive, carrying one standard through to completion and measuring the transmittancy before proceeding with the next.
3. Immediately set the zero concentration standard at 100% transmission at a wave length of 490 mμ and record the transmittancy of the 0.1, 0.2, 0.3 and 0.4 mg per cent standards. The transmittancy of the 0.4 mg standard should be approximately 36%.

4. Plot the observed values on semi-log graph paper—the points fall on a straight line which passes through the origin (Calibration Curve M-15).
5. Prepare a calibration table from the T-C values obtained from the plotted curve.

PROCEDURE FOR USE WITH LINEAR-SCALE PHOTOELECTRIC COLORIMETERS (SHEARD-SANFORD TYPE)

TEST PROCEDURE

Exactly as outlined; set the reagent blank at 100 and use a blue-green filter with maximum transmission at 490 mμ.

CALIBRATION PROCEDURE

Exactly as outlined—use a blue-green filter with maximum transmission at 490 mμ; plot the observed readings on semi-log graph paper—the points fall on a straight line passing through the origin.

PROCEDURE FOR USE WITH LOGARITHMIC-SCALE PHOTOELECTRIC COLORIMETERS (KLETT-SUMMERSON TYPE)

TEST PROCEDURE

Exactly as outlined; set the reagent blank at 0 and use a blue-green filter with maximum transmission at 490 mμ.

CALIBRATION PROCEDURE

Exactly as outlined—use a blue-green filter with maximum transmission at 490 mμ; plot the observed readings on linear graph paper—the points fall on a straight line passing through the origin.

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NOTES

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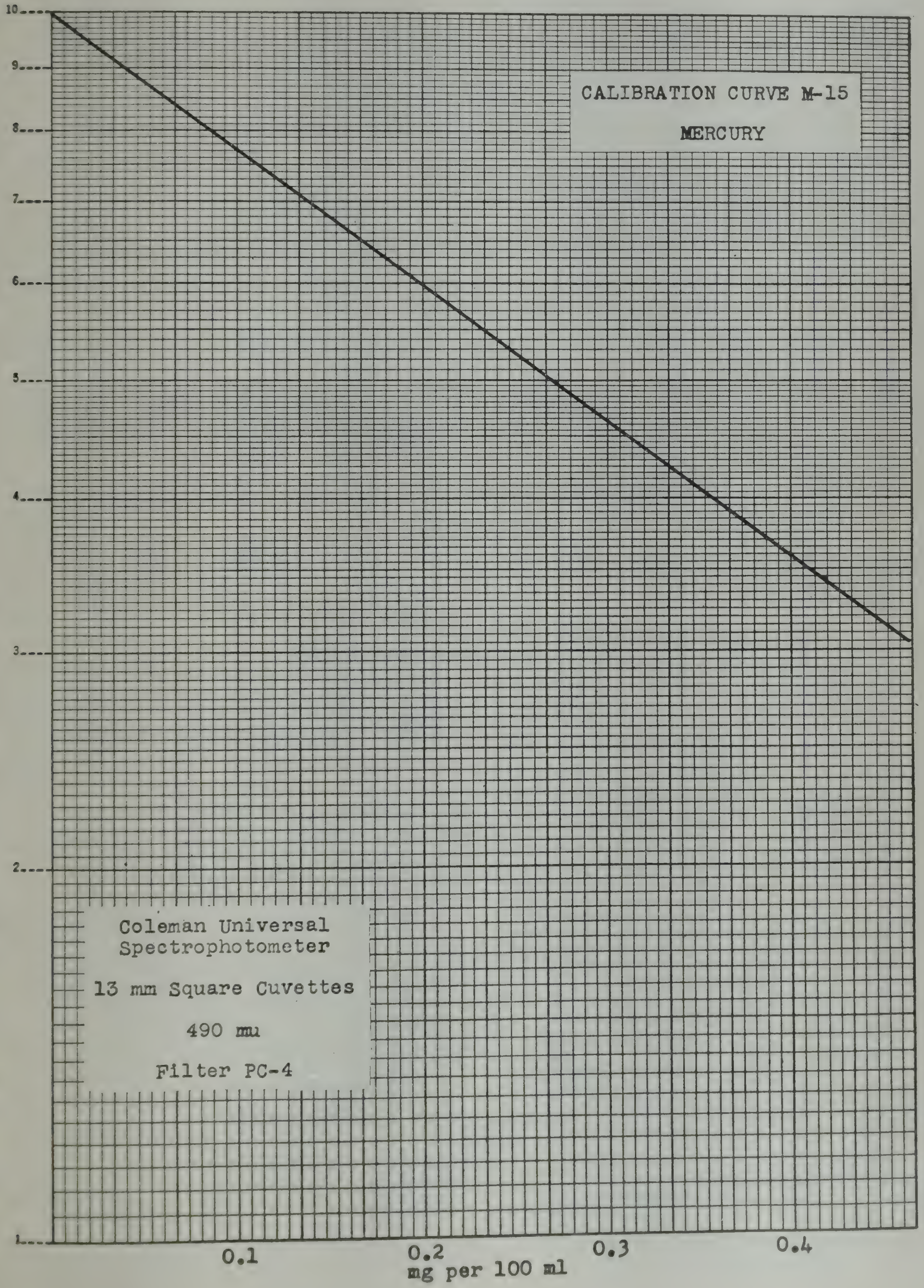
10
9
8
7
6
5
4
3
2
1
CALIBRATION CURVE M-15
MERCURY
Coleman Universal Spectrophotometer
13 mm Square Cuvettes
490 mu
Filter PC-4
0.1
0.2
0.3
0.4
mg per 100 ml

Meter Reading	0	1	2	3	4	5	6	7	8	9
0										
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50										
60										
70										
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90										

NICOTINIC ACID

A spectrophotometric adaptation based on the methods and modifications of Swaminathan (1938), Bandier and Hald (1939), Bandier (1939), Perlzweig, Levy and Sarett (1940), Dann and Handler (1941), Stotz (1941) and Klein, Perlzweig and Handler (1942)

PRINCIPLE

The protein-free filtrate is hydrolyzed to free the nicotinic acid which is then treated at a carefully controlled pH with cyanogen bromide in the presence of p-methylaminophenol to produce a yellow color (König reaction).

DETERMINATION IN RED BLOOD CELLS

SAMPLE

Blood withdrawn during post-absorptive state. Anticoagulant—potassium oxalate. Analyses on erythrocytes are preferable to whole blood determinations.

TEST PROCEDURE

Special Apparatus

Ostwald-Folin pipettes accurately calibrated "to contain" 2 ml.

Mixer-covers to fit tubes 3/4 to 1 inch in diameter as described by Klein, Perlzweig and Handler (Fig. N-10).

Graduated test tubes—25 ml in 0.5 ml; the 18 and 20 ml graduations must be accurately calibrated.

Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.

Reagents

1. **Sulfuric Acid, Volumetric 2/3 N.** Slowly add 20 ml concentrated sulfuric acid (H_2SO_4—Grasselli, A.C.S.) to 1000 ml distilled water and mix well; titrate against standard 1 N alkali using phenolphthalein as indicator; adjust accordingly and retitrate—20 ml of 2/3 N acid require 13.3 ml of 1 N alkali.
2. **Sodium Tungstate, 10% Aqueous.** Dissolve 10 gm sodium tungstate ($Na_2WO_4 \cdot 2H_2O$—Mallinckrodt, Folin Special) in distilled water and dilute to 100 ml. The solution must be neutral or slightly alkaline to phenolphthalein, not more than 0.4 ml being required to neutralize 10 ml 0.1 N hydrochloric acid. Preserve in resistant-glass bottle.
3. **Hydrochloric Acid, Concentrated** (HCl—Baker & Adamson, A.C.S.).
4. **Phenolphthalein, 0.5% Alcoholic.** Dissolve 500 mg phenolphthalein ($C_{20}H_{14}O_4$—Baker & Adamson, A.C.S.) in 100 ml absolute methyl alcohol (CH_3OH—Merck, A.C.S.).
5. **Potassium Hydroxide, 11 M.** In a 2000 ml Florence flask place 400 ml distilled water; add slowly and with constant shaking, 726 gm potassium hydroxide pellets (KOH—Baker & Adamson, A.C.S.); shake until dissolved and allow to stand until cool; transfer to a ceresin-lined bottle and allow to stand until sedimentation is complete; use only the clear supernatant fluid.
6. **Wide Range Indicator Paper** (Hydrion).

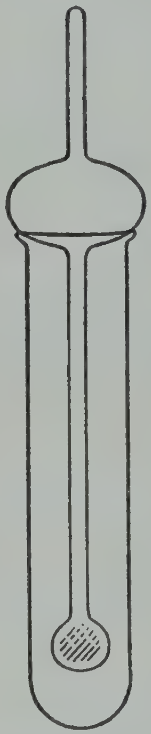
Fig. N-10.

7. **Hydrochloric Acid, 4 M.** Slowly add 343 ml concentrated hydrochloric acid (3) to 600 ml distilled water and mix; allow to cool and dilute to 1000 ml. Preserve in glass-stoppered bottle.
8. **Potassium Phosphate, Monobasic, 1.6 M.** Dissolve 21.7 gm monopotassium phosphate (KH_2PO_4—Baker & Adamson, A.C.S.) in distilled water and dilute to 100 ml.
9. **Cyanogen Bromide, 4% Aqueous.** In a 100 ml mixing cylinder place 4 gm crystalline cyanogen bromide (BrCN—Eastman #919); add distilled water to volume; shake until dissolved and adjust volume. Preserve in glass-stoppered bottle in refrigerator. Stability approximately one month.

   Note: THIS REAGENT IS EXTREMELY POISONOUS. The solution should always be dispensed from a burette and never pipetted. Extreme care must be used in handling the cyanogen bromide; all operations, including weighing, being carried out in a well ventilated hood or in such a manner that none of the fumes are inhaled. Since the crystals are highly corrosive, they must not be allowed to come into contact with the skin.
10. **p-Methylaminophenol Sulfate, Saturated Aqueous.** In a 50 ml mixing cylinder place 2.5 gm p-methylaminophenol sulfate ($(CH_3NHC_6H_4OH)_2 \cdot H_2SO_4$—Eastman #P615); add distilled water to volume and shake thoroughly; allow to settle for a few minutes and use the clear supernatant fluid. Discard when any trace of color becomes discernible. This reagent must be prepared fresh immediately before use.
11. **Sulfuric Acid, Volumetric 0.35 N.** Dilute 52.5 ml 2/3 N sulfuric acid (1) to 100 ml with distilled water.
12. **Potassium Phosphate-Nicotinic Acid Solution.** Dissolve 21.7 gm monopotassium phosphate (KH_2PO_4—Baker & Adamson, A.C.S.) in approximately 80 ml distilled water; add 1 ml nicotinic acid stock standard (Reagent #13a); mix

and dilute to volume with distilled water. Note: This solution is used only for low concentrations of nicotinic acid when an internal standard is required (see note under Technic).

Technic

Note: Unless all variables in the technic can be accurately controlled, it is advisable to run a standard (containing 0.01 mg nicotinic acid) simultaneously with the unknown, thus affording a convenient means by which a correction factor may be determined.

Filtration is not permissible at any point in the procedure.

For concentrations of nicotinic acid less than 5 micrograms per ml, use Reagent #12 in place of Reagent #8 and subtract 10 micrograms (nicotinic acid in 2 ml of Reagent #12) from the value obtained from the calibration table in Step 29.

1. Centrifuge 5 ml oxalated whole blood at 2000 rpm for 60 minutes or until the red cell volume is constant.
2. By means of a capillary-tipped pipette, draw off all of the supernatant plasma together with a few drops of the sedimented red cells and discard (or use for other analyses).
3. In a 20 × 150 mm Pyrex test tube place
   12 ml distilled water
   2 ml sedimented red cells from an Ostwald-Folin pipette, thoroughly rinsing out the pipette by drawing in and expelling some of the mixture several times.
4. Mix thoroughly and allow to stand for 10 minutes until hemolysis is complete.
5. Add 3 ml 2/3 N sulfuric acid (1) and mix.
6. Add 3 ml 10% sodium tungstate (2), drop by drop, while constantly rotating the tube.
7. Shake vigorously until foam-free.
8. Place the tube in boiling water for 20 minutes, mixing frequently during the first 5 minutes and then occasionally thereafter (this prevents the protein coagulum from collecting at the top of the tube with its consequent expulsion), keeping the tube covered at all times with a mixer-cover.
9. Remove mixer-cover and cool to room temperature under running tap water.
10. Centrifuge at 1800 rpm for 10 minutes; re-centrifuge, if necessary, until the centrifugate is crystal clear—do not filter.
11. In a 25 ml graduated test tube place
    10 ml clear centrifugate
    2 ml concentrated hydrochloric acid (3) and mix.
12. Place the tube in boiling water for 75 minutes with mixer-cover in place.
13. Cool to room temperature under running tap water, remove mixer-cover, washing off with a few drops of distilled water.
14. Add 1 drop 0.5% phenolphthalein (4).
15. Add 11 M potassium hydroxide (5), drop by drop, until just alkaline as indicated by a faint pink color.
16. Using wide range indicator paper (6), adjust to pH 5.0 by adding 4 M hydrochloric acid (7), drop by drop.
17. Add 2 ml 1.6 M monopotassium phosphate (8) and mix.

Note: For low concentrations requiring an internal standard, use Reagent #12 in place of Reagent #8—see note under technic.

18. Dilute to 18 ml with distilled water.
19. In a 25 ml graduated test tube (sample) place 9 ml hydrolysate (from Step 18).
20. In another 25 ml graduated test tube (reagent blank) place 9 ml distilled water.
21. Place both tubes in a water bath at 75–80° C. for 5 minutes.
22. To each tube add 1 ml freshly prepared 4% cyanogen bromide (9) and mix.
23. Replace tubes in water bath at 75–80° C. for 5 minutes.
24. Cool to room temperature in ice cold water.
25. Add 10 ml freshly prepared saturated p-methylaminophenol sulfate (10) and mix.
26. Adjust the volume in each tube to 20 ml with distilled water and mix by inversion.
27. In a third 25 ml graduated test tube (sample blank) place
    9 ml hydrolysate (from Step 18)
    1 ml 0.35 N sulfuric acid (11) and mix.
    10 ml distilled water and mix by inversion.
28. Allow all tubes to stand in the dark at room temperature for 60 minutes.
29. Measure the transmittancy of the sample against the reagent blank set at 100% transmission at a wave length of 400 mμ and obtain the value for nicotinic acid in micrograms per ml from the calibration table—record as result (a).
30. Measure the transmittancy of the sample blank against distilled water set at 100% transmission at a wave length of 400 mμ and obtain the nicotinic acid equivalent in micrograms per ml from the calibration table—record as result (b).

Calculation

Value for nicotinic acid in sample in micrograms per ml (result (a)) − Nicotinic acid equivalent in sample blank in micrograms per ml (result (b)) = Micrograms nicotinic acid per ml red blood cells

CALIBRATION PROCEDURE

Special Apparatus

Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.

Graduated test tubes—25 ml in 0.5 ml; the 20 ml graduation must be accurately calibrated.

Reagents

6. **Wide Range Indicator Paper.** Same as for Test Procedure.

7. **Hydrochloric Acid, 4 M.** Same as for Test Procedure.
9. **Cyanogen Bromide, 4% Aqueous.** Same as for Test Procedure.
10. **p-Methylaminophenol Sulfate, Saturated Aqueous.** Same as for Test Procedure.
13. **Nicotinic Acid Standards**
    (a) Nicotinic acid stock standard (1 ml contains 1 mg nicotinic acid). In a 100 ml volumetric flask place 100 mg nicotinic acid ($C_6H_5O_2N$—U.S.P. Reference Standard); dissolve in distilled water and dilute to volume. Preserve in refrigerator.
    (b) Dilute standard (1 ml contains 0.025 mg nicotinic acid). In an Erlenmeyer flask place slightly more than 100 ml distilled water and adjust to pH 5.0 by the addition of 4 M hydrochloric acid (7), delivered in small drops from a capillary-tipped dropper, using wide range indicator paper (6). In a 100 ml volumetric flask place 2.5 ml stock standard (13a) and dilute to volume with the freshly adjusted distilled water. Prepare just before use.
    (c) Calibration standards. In a series of six 10 ml graduated test tubes place respectively 0, 1, 2, 3, 4 and 5 ml of the dilute standard (13b); add 5, 4, 3, 2, 1 and 0 ml distilled water respectively, bringing the volume in each tube to 5 ml. These standards represent concentrations of nicotinic acid equivalent to 0, 10, 20, 30, 40 and 50 microgram per ml or 0, 1, 2, 3, 4 and 5 mg per 100 ml sample. Prepare just before use and proceed immediately as follows.

Technic

1. Transfer 1 ml of each calibration standard (13c) to a correspondingly marked 25 ml graduated test tube.
2. To each tube add 8 ml distilled water and mix.
3. Place the tubes in a water bath at 75–80° C. for 5 minutes.
4. Add 1 ml freshly prepared 4% cyanogen bromide (9) and mix.
5. Replace tubes in water bath at 75–80° C. for 5 minutes.
6. Cool to room temperature in ice cold water.
7. Add 10 ml freshly prepared saturated p-methylaminophenol sulfate (10) and mix.
8. Adjust the volume in each tube to 20 ml with distilled water and mix by inversion.
9. Allow to stand in the dark at room temperature for 60 minutes.
10. Set the zero concentration standard at 100% transmission at a wave length of 400 mμ and record the transmittancy of the 10, 20, 30, 40 and 50 microgram per ml standards. The transmittancy of the 50 microgram standard should be approximately 42%.
11. Plot the observed values on semi-log graph paper—the points fall on a straight line which passes through the origin (Calibration Curve N-10).
12. Prepare a calibration table from the T-C values obtained from the plotted curve.

PROCEDURE FOR USE WITH LINEAR-SCALE PHOTOELECTRIC COLORIMETERS (SHEARD-SANFORD TYPE)

TEST PROCEDURE

Exactly as outlined; set the blanks at 100 and use a blue filter.

CALIBRATION PROCEDURE

Exactly as outlined—use a blue filter; plot the observed readings on semi-log graph paper—the points fall on a straight line passing through the origin.

PROCEDURE FOR USE WITH LOGARITHMIC-SCALE PHOTOELECTRIC COLORIMETERS (KLETT-SUMMERSON TYPE)

TEST PROCEDURE

Exactly as outlined; set the blanks at 0 and use a blue filter.

CALIBRATION PROCEDURE

Exactly as outlined—use a blue filter; plot the observed readings on linear graph paper—the points fall on a straight line passing through the origin.

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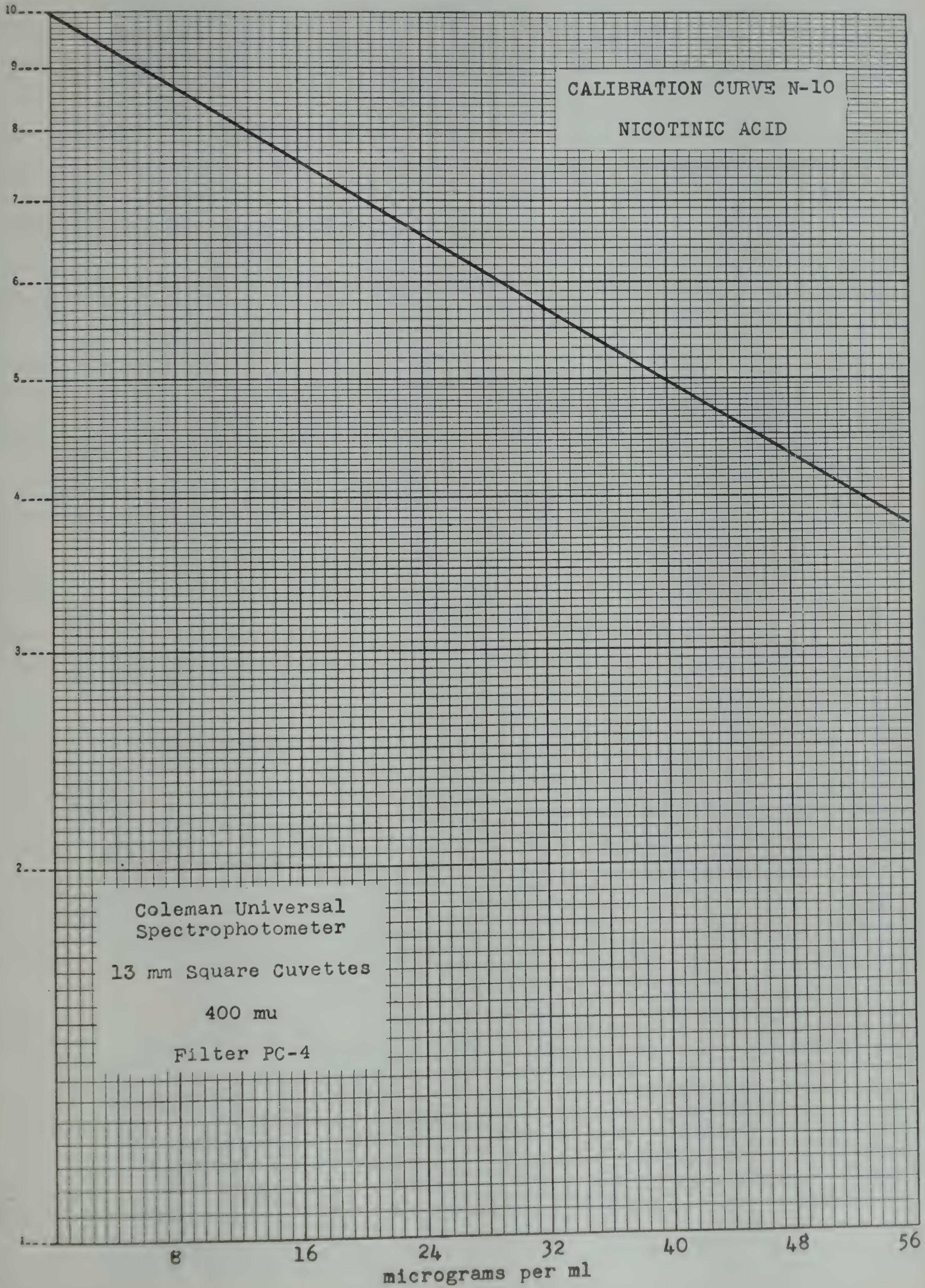
NOTES


CALIBRATION CURVE N-10
NICOTINIC ACID
Coleman Universal Spectrophotometer
13 mm Square Cuvettes
400 mu
Filter PC-4
10
9
8
7
6
5
4
3
2
1
8
16
24
32
40
48
56
micrograms per ml

Meter Reading	0	1	2	3	4	5	6	7	8	9
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10										
20										
30										
40										
50										
60										
70										
80										
90										

NICOTINIC ACID DERIVATIVES

A spectrophotometric adaptation based on the methods and modifications of Swaminathan (1938), Bandier and Hald (1939), Bandier (1939), Perlzweig, Levy and Sarett (1940), Dann and Handler (1941), Stotz (1941) and Klein, Perlzweig and Handler (1942)

PRINCIPLE

Urine is subjected to acid hydrolysis to convert all nicotinic acid derivatives (except trigonelline) into nicotinic acid which is then adsorbed on Lloyd's reagent. After elution, lead nitrate and potassium phosphate are used to remove pigments and other interfering substances and the nicotinic acid is determined as outlined in the method for its analysis in red blood cells (Method N-10), the same analytical principles being involved. Trigonelline is converted to nicotinic acid by heating the acid hydrolysate with strong alkali in the presence of urea before adsorption on Lloyd's reagent. Procedure A determines all nicotinic acid with the exception of trigonelline; Procedure B determines the same nicotinic acid derivatives and in addition 70% of nicotinic acid color produced by the trigonelline.

DETERMINATION IN URINE

SAMPLE

Aliquot of a 24-hour specimen. Keep cool during collection—preserve with thiophene-free toluene. The nicotinic acid content remains stable for several days in refrigerator. When analyses are to be made, patients must abstain from smoking and no pyridine drugs must be administered.

TEST PROCEDURE

Special Apparatus

Centrifuge tubes, 100 ml.
Kjeldahl flasks, 100 ml, Pyrex, with Hopkins condenser to fit.
Graduated test tubes—25 ml in 0.5 ml; the 15 and 20 ml graduations must be accurately calibrated.
Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.

Reagents

1. **Phenolphthalein, 0.5% Alcoholic.** Dissolve 500 mg phenolphthalein ($C_{20}H_{14}O_4$—Baker & Adamson, A.C.S.) in 100 ml absolute methyl alcohol (CH_3OH—Merck, A.C.S.).
2. **Potassium Hydroxide, Volumetric 0.5 N.** Dilute 4 ml 12 N potassium hydroxide (Reagent #5) to 96 ml with distilled water. Preserve in ceresin-lined bottle.
3. **Hydrochloric Acid, Concentrated** (HCl—Baker & Adamson, A.C.S.).
4. **Nitric Acid, Concentrated** (HNO_3—Grasselli, A.C.S.).
5. **Potassium Hydroxide, Volumetric 12 N.** In a 2000 ml Florence flask place 500 ml distilled water; add slowly and with constant shaking, 800 gm potassium hydroxide pellets (KOH—Baker & Adamson, A.C.S.); shake until dissolved and allow to stand until cool; after sedimentation is complete, dilute 10 ml of the approximately 12 N clear supernatant fluid to 100 ml; titrate against standard 1 N acid using phenolphthalein as indicator; adjust accordingly and re-titrate—24 ml of 1 N acid require 20 ml of a 1:10 dilution of 12 N alkali. Preserve in ceresin-lined bottle.
6. **Wide Range Indicator Paper** (Hydrion).
7. **Sulfuric Acid, Volumetric 0.2 N.** Dilute 10 ml 2 N sulfuric acid (Reagent #16) to 100 ml with distilled water.
8. **Lloyd's Reagent** (Lilly).
9. **Lead Nitrate, Powder.** Finely pulverized crystalline lead nitrate ($Pb(NO_3)_2$—Baker & Adamson, A.C.S.).
10. **Ammonium Hydroxide, Concentrated, 28%** (Baker & Adamson, A.C.S.).
11. **Potassium Phosphate, Tribasic, Powder.** Finely pulverized crystalline tripotassium phosphate (K_3PO_4—Baker & Adamson, Reagent).
12. **Phosphoric Acid, Volumetric 5 N.** Dilute 200 ml 85% phosphoric acid (H_3PO_4—Baker & Adamson, A.C.S.) to 1000 ml with distilled water and mix; allow to cool; dilute 2 ml of this approximately 5 N solution to 100 ml; titrate against standard 0.1 N alkali using phenolphthalein as indicator; adjust accordingly and re-titrate—20 ml of a 1:50 dilution of 5 N acid require 20 ml of 0.1 N alkali. Preserve in glass-stoppered bottle.
13. **Potassium Phosphate, Monobasic, Powder.** Finely pulverized crystalline monopotassium phosphate (KH_2PO_4—Baker & Adamson, A.C.S.).
14. **Cyanogen Bromide, 4% Aqueous.** In a 100 ml mixing cylinder place 4 gm crystalline cyanogen bromide ($BrCN$—Eastman #919); add distilled water to volume; shake until dissolved and adjust volume. Preserve in glass-stoppered bottle in refrigerator. Stability approximately one month.

Note: THIS REAGENT IS EXTREMELY POISONOUS. The solution should always be dispensed from a burette and never pipetted. Extreme care must be used in handling the cyanogen bromide; all operations, including weighing, being carried out in a well ventilated hood or in such a manner that none of the fumes are inhaled. Since the crystals are highly corrosive, they must not be allowed to come into contact with the skin.

15. **p-Methylaminophenol Sulfate, Saturated Aqueous.** In a 50 ml mixing cylinder place 2.5

gm p-methylaminophenol sulfate ($(CH_3NHC_6H_4OH)_2 \cdot H_2SO_4$—Eastman #P615); add distilled water to volume and shake thoroughly; allow to settle for a few minutes and use the clear supernatant fluid. Discard when any trace of color becomes discernible. This reagent must be prepared fresh immediately before use.

16. **Sulfuric Acid, Volumetric 2 N.** Slowly add 60 ml concentrated sulfuric acid (H_2SO_4—Grasselli, A.C.S.) to 1000 ml distilled water and mix well; allow to cool; dilute 10 ml of this approximately 2 N solution to 100 ml; titrate against standard 0.1 N alkali using phenolphthalein as indicator; adjust accordingly and re-titrate—10 ml of a 1:10 dilution of 2 N acid require 20 ml of 0.1 N alkali.
17. **Urea** ($(NH_2)_2CO$—Merck, Reagent).

Technic

A. Total Nicotinic Acid Derivatives Exclusive of Trigonelline

Note: Unless all variables in the technic can be accurately controlled, it is advisable to run a standard (containing 0.015 mg nicotinic acid) simultaneously with the unknown, thus affording a convenient means by which a correction factor may be determined.

Filtration is not permissible at any point in the procedure.

1. Centrifuge about 100 ml thoroughly mixed urine at 1500 rpm for 3 minutes.
2. In a 150 ml beaker place 20 ml centrifuged urine and 1 drop 0.5% phenolphthalein (1).
3. Add 0.5 N potassium hydroxide (2), drop by drop from a capillary-tipped dropper, until just alkaline as indicated by a faint pink color.
4. Evaporate on a steam bath to a thick syrup.
5. Quantitatively transfer the syrupy liquid to a 100 ml Pyrex Kjeldahl flask, rinsing out the beaker with 4 ml distilled water followed by 6 ml concentrated hydrochloric acid (3).
6. Add 0.25 ml concentrated nitric acid (4) and several glass beads.
7. Place a Hopkins condenser in the mouth of the flask and heat gently over a micro burner for 60 minutes—apply heat gradually until foaming ceases, then use full flame; the solution darkens but after 15 minutes becomes light yellow.
8. Allow to cool to room temperature.
9. Transfer the hydrolysate to a 25 ml mixing cylinder, rinsing out the flask with successive portions of distilled water until the volume in the cylinder is exactly 20 ml.
10. Transfer 15 ml to a 50 ml beaker and add 2 ml 12 N potassium hydroxide (5).
11. Allow to cool and adjust to pH 1.0 by the dropwise addition of 0.5 N potassium hydroxide (2), using wide range indicator paper (6).
12. Transfer to a 22 × 175 mm test tube, rinsing out the beaker with 0.2 N sulfuric acid (7) from a wash bottle.
13. Add 2 gm Lloyd's reagent (8), insert a rubber stopper and shake vigorously several times—release gas pressure, re-stopper and shake vigorously for 1 minute by inversion.
14. Rinse off stopper into the tube and wash down walls with 0.2 N sulfuric acid (7).
15. Centrifuge at 1500 rpm for 3 minutes and discard the supernatant fluid.
16. Add 10 ml 0.2 N sulfuric acid (7) to the sedimented Lloyd's reagent, mix thoroughly, centrifuge at 1500 rpm for 3 minutes and discard the supernatant fluid.
17. Repeat Step 16.
18. Add 8 ml 0.5 N potassium hydroxide (2) to the sedimented Lloyd's reagent and shake for 1 minute.
19. Add 5 ml distilled water and centrifuge at 1800 rpm for 5 minutes.
20. Quantitatively transfer the colored supernatant fluid to an accurately graduated 25 ml test tube and dilute to 15 ml with distilled water.
21. Add 600 mg powdered lead nitrate (9) and shake thoroughly—if the liquid is still highly colored, add 2 drops ammonium hydroxide (10) and shake again.
22. Centrifuge at 1500 rpm for 3 minutes—the fluid should now be light yellow in color.
23. Decant the supernatant fluid into an 18 × 150 mm test tube and add 200 mg powdered tripotassium phosphate (11)—the solution must be definitely alkaline—if not, add more of the phosphate.
24. Allow to stand for 10 minutes and centrifuge at 1500 rpm for 3 minutes.
25. Transfer 5 ml of the clear supernatant fluid to an accurately graduated 25 ml test tube (sample) and adjust to pH 5.0 by the dropwise addition of 5 N phosphoric acid (12), using wide range indicator paper (6).
26. In another accurately graduated 25 ml test tube (reagent blank) place 100 mg monopotassium phosphate (13); add 5 ml distilled water and shake until dissolved.
27. Dilute the contents of both tubes to 9 ml with distilled water.
28. Place both tubes in a water bath at 75–80° C. for 5 minutes.
29. To each tube add 1 ml freshly prepared 4% cyanogen bromide (14) and mix.
30. Replace tubes in water bath at 75–80° C. for 5 minutes.
31. Cool to room temperature in ice cold water.
32. Add 10 ml freshly prepared saturated p-methylaminophenol sulfate (15) and mix.
33. Adjust the volume in each tube to 20 ml with distilled water and mix by inversion.

34. In a third 25 ml graduated test tube (sample blank) place
    5 ml clear supernatant fluid (from Step 24) and adjust to pH 5.0 by the dropwise addition of 5 N phosphoric acid (12), using wide range indicator paper (6)
    0.18 ml 2 N sulfuric acid (16) and dilute to 20 ml with distilled water.
35. Allow all tubes to stand in the dark at room temperature for 60 minutes.
36. Measure the transmittancy of the sample against the reagent blank set at 100% transmission at a wave length of **400** $m\mu$ and obtain the value for nicotinic acid in micrograms per ml from the calibration table—record as result (a).
37. Measure the transmittancy of the sample blank against distilled water set at 100% transmission at a wave length of **400** $m\mu$ and obtain the nicotinic acid equivalent in micrograms per ml from the calibration table—record as result (b).

Calculation

Value for nicotinic acid in sample in micrograms per ml (result (a)) − Nicotinic acid equivalent in sample blank in micrograms per ml (result (b)) = Micrograms nicotinic acid per ml urine

$$\frac{\text{Volume of 24-hour specimen} \times \text{Nicotinic acid in micrograms per ml}}{1000} = \text{Mg nicotinic acid excreted in 24 hours}$$

Method N-11b

B. Total Nicotinic Acid Derivatives Plus Trigonelline

1. Centrifuge about 100 ml thoroughly mixed urine at 1500 rpm for 3 minutes.
2. Proceed exactly as outlined in Procedure A, Steps 2–9 inclusive.
3. In a 22 × 175 mm test tube place
    300 mg urea (17)
    1.3 ml 12 N potassium hydroxide (5).
4. Place the tube in a water bath at 75–80° C. for 5 minutes—keep the tube closed with a small funnel.
5. Add 1 ml acid hydrolysate and mix.
6. Cover tube and replace in water bath at 75–80° C. for 45 minutes.
7. Quantitatively transfer the contents of the tube to a 50 ml beaker with 8–10 ml 0.2 N sulfuric acid (7).
8. Allow to cool and adjust to pH 1.0 by the dropwise addition of concentrated hydrochloric acid (3), using wide range indicator paper (6)—1.5–2.0 ml are required.
9. Proceed exactly as outlined in Procedure A, Steps 12–35 inclusive.
10. Measure the transmittancy of the sample against the reagent blank set at 100% transmission at a wave length of **400** $m\mu$ and multiply the value from the calibration table by 15 to obtain the concentration of nicotinic acid in micrograms per ml—record as result (c).
11. Measure the transmittancy of the sample blank against distilled water set at 100% transmission at a wave length of **400** $m\mu$ and multiply the value from the calibration table by 15 to obtain the nicotinic acid equivalent in micrograms per ml—record as result (d).

Calculation

Value for nicotinic acid in sample in micrograms per ml (result (c)) − Nicotinic acid equivalent in sample blank in micrograms per ml (result (d)) = Micrograms nicotinic acid per ml urine due to nicotinic acid derivatives plus trigonelline

$$(\text{Micrograms nicotinic acid due to nicotinic acid derivatives plus trigonelline} - \text{Micrograms nicotinic acid derivatives exclusive of trigonelline}) \times \frac{100}{70} = \text{Micrograms trigonelline expressed as nicotinic acid}$$

CONVERSION FACTOR

Micrograms trigonelline in terms of nicotinic acid × 1.11 = Trigonelline in micrograms per ml urine

CALIBRATION PROCEDURE

Special Apparatus

Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.

Graduated test tubes—25 ml in 0.5 ml; the 20 ml graduation must be accurately calibrated.

Reagents

6. **Wide Range Indicator Paper.** Same as for Test Procedure.
12. **Phosphoric Acid, Volumetric 5 N.** Same as for Test Procedure.

14. **Cyanogen Bromide, 4% Aqueous.** Same as for Test Procedure.
15. **p-Methylaminophenol Sulfate, Saturated Aqueous.** Same as for Test Procedure.
16. **Sulfuric Acid, Volumetric 2 N.** Same as for Test Procedure.
18. **Nicotinic Acid Standards**
    (a) Nicotinic acid stock standard (1 ml contains 1 mg nicotinic acid). In a 100 ml volumetric flask place 100 mg nicotinic acid ($C_6H_5O_2N$—U.S.P. Reference Standard); dissolve in distilled water and dilute to volume. Preserve in refrigerator.
    (b) Dilute standard (1 ml contains 0.03 mg nicotinic acid). In an Erlenmeyer flask place slightly more than 100 ml distilled water and adjust to pH 5.0 by the addition of 5 N phosphoric acid (12), delivered in small drops from a capillary-tipped dropper, using wide range indicator paper (6). In a 100 ml volumetric flask place 3 ml stock standard (18a) and dilute to volume with the freshly adjusted distilled water. Prepare just before use.
    (c) Calibration standards. In a series of six 10 ml graduated test tubes place respectively 0, 1, 2, 3, 4 and 5 ml of the dilute standard (18b); add 5, 4, 3, 2, 1 and 0 ml distilled water respectively, bringing the volume in each tube to 5 ml. These standards represent concentrations of nicotinic acid equivalent to 0, 1.2, 2.4, 3.6, 4.8 and 6.0 micrograms per ml or 0, 0.12, 0.24, 0.36, 0.48 and 0.6 mg per 100 ml sample. Prepare just before use and proceed immediately as follows.

Technic

1. Transfer 1 ml of each calibration standard (18c) to a correspondingly marked 25 ml graduated test tube.
2. To each tube add 8 ml distilled water and mix.
3. Place the tubes in a water bath at 75–80° C. for 5 minutes.
4. Add 1 ml freshly prepared 4% cyanogen bromide (14) and mix.
5. Replace tubes in water bath at 75–80° C. for 5 minutes.
6. Cool to room temperature in ice cold water.
7. Add 10 ml freshly prepared saturated p-methylaminophenol sulfate (15) and mix.
8. Adjust the volume in each tube to 20 ml with distilled water and mix by inversion.
9. Allow to stand in the dark at room temperature for 60 minutes.
10. Set the zero concentration standard at 100% transmission at a wave length of 400 mμ and record the transmittancy of the 1.2, 2.4, 3.6, 4.8, and 6.0 micrograms per ml standards. The transmittancy of the 6 microgram standard should be approximately 35%.
11. Plot the observed values on semi-log graph paper—the points fall on a straight line which passes through the origin (Calibration Curve N-11).
12. Prepare a calibration table from the T-C values obtained from the plotted curve.

PROCEDURE FOR USE WITH LINEAR-SCALE PHOTOELECTRIC COLORIMETERS (SHEARD-SANFORD TYPE)

TEST PROCEDURE

Exactly as outlined; set the blanks at 100 and use a blue filter.

CALIBRATION PROCEDURE

Exactly as outlined—use a blue filter; plot the observed readings on semi-log graph paper—the points fall on a straight line passing through the origin.

PROCEDURE FOR USE WITH LOGARITHMIC-SCALE PHOTOELECTRIC COLORIMETERS (KLETT-SUMMERSON TYPE)

TEST PROCEDURE

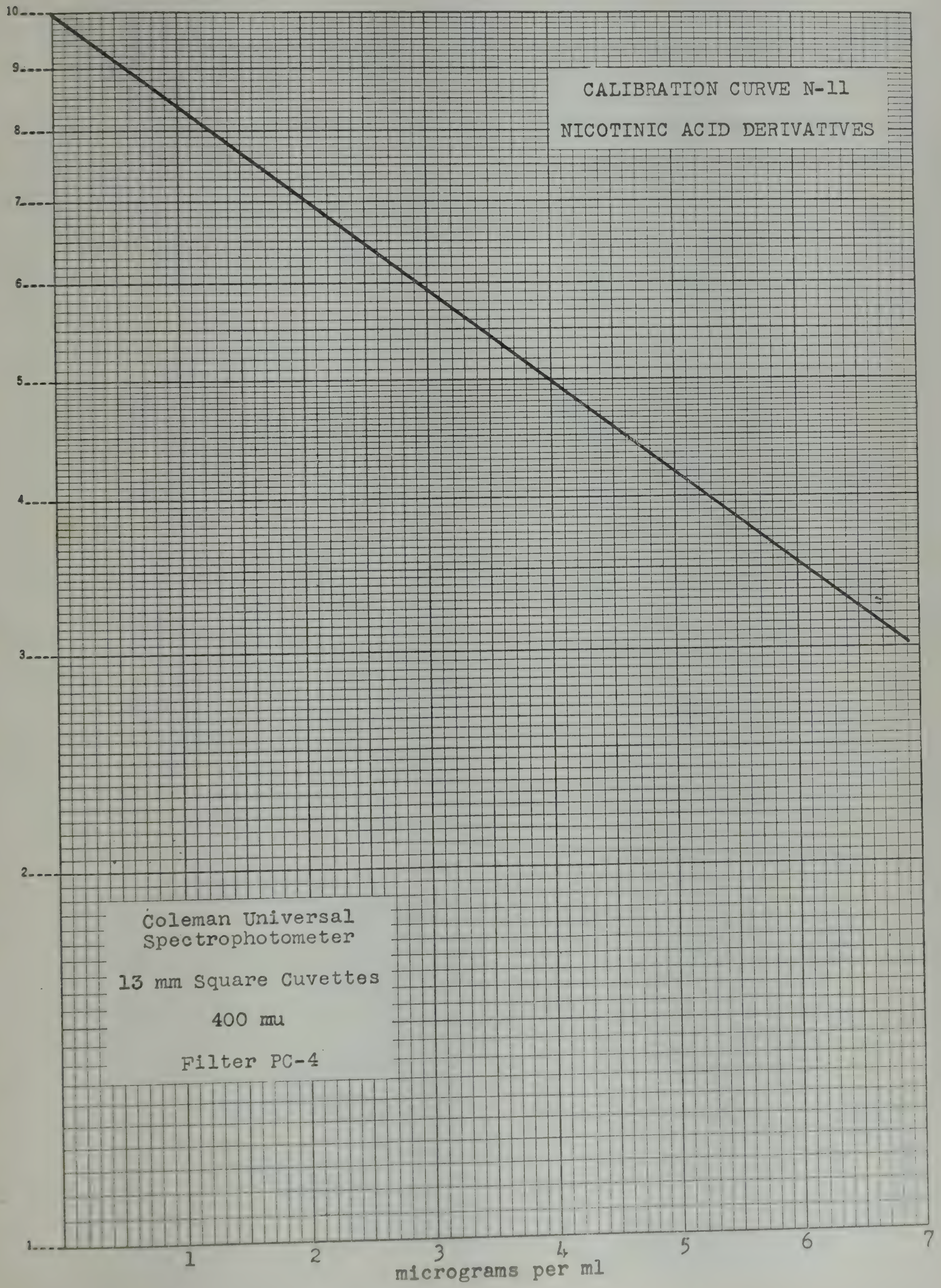
Exactly as outlined; set the blanks at 0 and use a blue filter.

CALIBRATION PROCEDURE

Exactly as outlined—use a blue filter; plot the observed readings on linear graph paper—the points fall on a straight line passing through the origin.

BIBLIOGRAPHY

Same as listed under Nicotinic Acid (Method N-10).


CALIBRATION CURVE N-11
NICOTINIC ACID DERIVATIVES
Coleman Universal Spectrophotometer
13 mm Square Cuvettes
400 mu
Filter PC-4
10
9
8
7
6
5
4
3
2
1
1
2
3
4
5
6
7
micrograms per ml

Meter Reading	0	1	2	3	4	5	6	7	8	9
0										
10										
20										
30										
40										
50										
60										
70										
80										
90										

NONPROTEIN NITROGEN

A spectrophotometric adaptation based on the methods and modifications of Folin and Wu (1919), Myers (1921), Campbell and Hanna (1937) and Hoffman and Osgood (1940)

PRINCIPLE

A trichloracetic acid protein-free filtrate is digested with a selenium-sulfuric acid mixture with the aid of hydrogen peroxide to convert nonprotein nitrogenous constituents to ammonium sulfate in which the ammonia is determined by means of the reddish-orange color developed with alkaline potassium mercuric iodide (Nessler reaction).

DETERMINATION IN WHOLE BLOOD, PLASMA OR SERUM

SAMPLE

Blood withdrawn during post-absorptive state. Anticoagulant—potassium oxalate. Contamination by ammonia or its salts must be avoided.

TEST PROCEDURE

Special Apparatus

Ostwald-Folin pipettes accurately calibrated "to contain" 1 ml.

Fister digestion tubes—Pyrex; 25 × 200 mm; glass-stoppered; the 25 ml graduation must be accurately calibrated. These tubes prevent contamination during mixing as well as protect the hands from the action of the alkali. Folin-Wu tubes may be used provided that they are accurately calibrated at 25 ml.

Micro burner, Argand type.

Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.

Reagents

1. **Water, Distilled, Ammonia-Free.** In an all-glass Pyrex distillation apparatus place 1 gm sodium carbonate (Na_2CO_3—Baker & Adamson, A.C.S.) and 1 gm potassium permanganate ($KMnO_4$—Baker & Adamson, A.C.S.); add 500 ml distilled water; redistil, rejecting the first 100 ml portion and collecting only the next 300 ml. Use for the preparation of all reagents and standards and where indicated in the test procedure.
2. **Trichloracetic Acid, 5% Aqueous.** Dilute 5 ml concentrated trichloracetic acid (a) to 100 ml with ammonia-free distilled water (1). Prepare fresh weekly.
   (a) Trichloracetic acid, concentrated; dissolve 100 gm crystalline trichloracetic acid (CCl_3-COOH—J. T. Baker, CP Analyzed) in ammonia-free distilled water (1) and dilute to 100 ml. Preserve in Pyrex glass-stoppered bottle. Stability indefinite.
3. **Selenium-Sulfuric Acid Digestion Mixture "A" (Fister).** In a 1000 ml Florence flask place 400 ml ammonia-free distilled water (1); add slowly, while cooling the flask under running tap water, 74.2 ml concentrated sulfuric acid (H_2SO_4—Grasselli, A.C.S.); cool to room temperature; transfer to a 500 ml mixing cylinder; add 0.15 ml selenium oxychloride ($SeOCl_2$—J. T. Baker); dilute to volume with ammonia-free distilled water and mix thoroughly. Preserve in Pyrex glass-stoppered bottle. Note: This is not the same digestion mixture used in the total nitrogen and albumin determinations.
4. **Hydrogen Peroxide, 30%** (H_2O_2—Merck, Reagent Superoxol, Low-Nitrogen).
5. **Nessler Reagent (Folin-Wu).** In a 1000 ml mixing cylinder place 700 ml 2.5 N sodium hydroxide (a); add 150 ml double iodide solution (b); dilute to volume with ammonia-free distilled water (1) and mix; allow to stand for three days to permit sedimentation of the small amount of precipitate which forms. Preserve in ceresin-lined bottle. Stability indefinite. Note: The alkaline concentration of this reagent is 7%—11.0–11.5 ml must be required to neutralize 20 ml 1 N hydrochloric acid, using phenolphthalein as indicator.
   (a) Sodium hydroxide, volumetric 2.5 N; prepare by diluting slightly more than the required amount of concentrated carbonate-free sodium hydroxide solution (see Appendix); dilute 20 ml of this approximately 2.5 N solution to 100 ml; titrate against standard 0.5 N acid using phenolphthalein as indicator; adjust accordingly and retitrate—20 ml of 0.5 N acid require 20 ml of a 1:5 dilution of 2.5 N alkali. Preserve in ceresin-lined bottle.
   (b) Double iodide solution (Folin-Wu); in a 500 ml Florence flask place 150 gm granular potassium iodide (KI—Mallinckrodt, A.C.S.) and 100 gm resublimed iodine (I—Baker & Adamson, A.C.S.); add 100 ml ammonia-free distilled water (1) and shake until the iodine has completely dissolved; add 150 gm redistilled metallic mercury (Hg—Baker & Adamson, A.C.S.); shake the flask continuously and vigorously until the red color of the iodine solution has become visibly pale, though still red; immediately start cooling under running tap water, continuing vigorous shaking, until the reddish color has been replaced by the greenish-yellow color of the double iodide. If properly prepared, the solution is clear

and there is a residue of shining metallic mercury; solutions showing a gray-green sludge are unsatisfactory. The whole operation should consume no more than 10–15 minutes. Since the reaction develops considerable heat, precautions should be taken to protect the hands during the shaking procedure. Decant the solution into a 2000 ml mixing cylinder, washing mercury and flask with liberal quantities of ammonia-free distilled water and decanting the washings into the cylinder. Dilute to volume with ammonia-free distilled water.

Technic

Note: For concentrations of nonprotein nitrogen in excess of 100 mg per 100 ml, use 0.5 ml sample in Step 1 and multiply the values obtained from the calibration table by 2.

1. In a 15 ml centrifuge tube place
   4 ml ammonia-free distilled water (1)
   1 ml sample from an Ostwald-Folin pipette, thoroughly rinsing out the pipette by drawing in and expelling some of the mixture several times.
2. Add 5 ml 5% trichloracetic acid (2), drop by drop, with constant agitation of the tube.
3. Insert a clean rubber stopper in the tube and shake vigorously for 30 seconds.
4. Allow to stand at room temperature for 10 minutes.
5. Centrifuge at 1500 rpm for 3 minutes—if the supernatant fluid is not crystal clear, filter through a small Whatman #40 paper.
6. In a special dry digestion tube (sample) place
   0.5 ml clear supernatant centrifugate
   2.5 ml selenium-sulfuric acid digestion mixture "A" (3)
   1 solid glass bead.
7. Clamp tube in a vertical position on a support and apply gentle heat from an Argand micro burner in such a manner that the flame at no time touches the tube above the level of the liquid.

Note: Carry out Steps 7–13 inclusive in a fume hood.

8. After boiling has started gradually increase the heat and place burner in upright position so that the top is about $^3/_4$ inch below the bottom of the tube and the point of the flame directly under the tube—the bubbles should at no time rise above the middle of the tube.
9. Heat until white fumes of SO_3 fill about one-half of the tube—none of these fumes should escape.
10. Insert a small funnel into the mouth of the tube and continue gentle heating with reduced flame, until the charred digest clears.
11. Remove the flame and allow to cool for 90 seconds.
12. Remove the funnel and allow 1 drop 30% hydrogen peroxide (4) to fall directly on the digest.
13. Replace funnel and heat gently for 2 minutes making certain that any charred material which may have adhered to the walls of the tube has been washed down and decomposed—the digest should be perfectly clear—if not, add another drop of hydrogen peroxide in the same manner and heat again until clear.
14. Without removing the funnel, allow to cool for 2 minutes and then place the tube in a beaker half-full of ice-cold water.
15. In another special digestion tube (reagent blank) place 2.5 ml selenium-sulfuric acid digestion mixture "A" (3) and place in the cold water bath with the other tube.
16. After 10 minutes in the cold water bath, add about 10 ml ammonia-free distilled water (1) to each tube, rinsing off the funnel in the sample tube at the same time and mix, allowing the tubes to remain in the bath.
17. Without removing the tubes from the bath add 10 ml Nessler reagent (5), allowing the reagent to fall directly into the solution.
18. Remove the tubes from the bath, dilute to 25 ml with ammonia-free distilled water (1), insert the glass stoppers and mix by inversion.
19. Replace the tubes in the bath and allow to remain for 5 minutes.
20. Remove the tubes from the bath and allow to stand at room temperature (21° C.) for 10 minutes.
21. Measure the transmittancy of the sample against the reagent blank set at 100% transmission at a wave length of **415** $m\mu$ and obtain the concentration of nonprotein nitrogen from the calibration table.

CALIBRATION PROCEDURE

Special Apparatus

Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.

Fister digestion tubes, glass-stoppered as described in the Test Procedure.

Micro burner, Argand type.

Reagents

1. **Water, Distilled, Ammonia-Free.** Same as for Test Procedure.
2. **Trichloracetic Acid, 5% Aqueous.** Same as for Test Procedure.
3. **Selenium-Sulfuric Acid Digestion Mixture "A" (Fister).** Same as for Test Procedure.
4. **Hydrogen Peroxide, 30%.** Same as for Test Procedure.
5. **Nessler Reagent (Folin-Wu).** Same as for Test Procedure.
6. **Nonprotein Nitrogen Standards**
   (a) Nitrogen stock standard (1 ml contains 10 mg nitrogen). Dry pyridine-free ammonium sulfate ($(NH_4)_2SO_4$ —Reagent Chemi-

cals, Special) at 110° C. for 1 hour; transfer 4.7166 gm to a 100 ml volumetric flask; add approximately 50 ml ammonia-free distilled water (1); shake until dissolved and dilute to volume with same. Preserve with a few drops of toluene. Stability indefinite.

(b) Dilute standard (1 ml contains 1 mg nitrogen). In a 100 ml volumetric flask place 10 ml stock standard (6a) and dilute to volume with ammonia-free distilled water (1). Prepare fresh daily.

(c) Calibration standards. In a series of six accurately calibrated 10 ml volumetric flasks place respectively 0, 2, 4, 6, 8 and 10 ml of the dilute standard (6b) and dilute to volume with ammonia-free distilled water (1). These standards represent concentrations of nonprotein nitrogen equivalent to 0, 20, 40, 60, 80 and 100 mg per 100 ml sample. Prepare just before use.

Technic

1. Treat 1 ml of each calibration standard (6c) exactly as outlined in the Test Procedure, Steps 1–20 inclusive—the standards are run simultaneously through Steps 1–6 inclusive, then consecutively through Steps 7–14 inclusive, Step 15 is omitted (since this duplicates the zero concentration calibration standard) and then simultaneously through Steps 16–20 inclusive.
2. Set the zero concentration standard at 100% transmission at a wave length of 415 mμ and record the transmittancy of the 20, 40, 60, 80 and 100 mg per cent standards. The transmittancy of the 100 mg standard should be approximately 35%.
3. Plot the observed values on semi-log graph paper—the points fall on a very slight curve which passes through the concentration axis slightly to the right of the origin (Calibration Curve N-15).
4. Prepare a calibration table from the T-C values obtained from the plotted curve.

Method N-15b

DETERMINATION IN URINE

SAMPLE

Aliquot of a 24-hour specimen. Keep cool during collection.

TEST PROCEDURE

Special Apparatus

As listed under the Test Procedure for blood.

Reagents

1. **Water, Distilled, Ammonia-Free.** Same as for blood.
2. **Trichloracetic Acid, 5% Aqueous.** Same as for blood.
3. **Selenium-Sulfuric Acid Digestion Mixture "A" (Fister).** Same as for blood.
4. **Hydrogen Peroxide, 30%.** Same as for blood.
5. **Nessler Reagent (Folin-Wu).** Same as for blood.

Technic

Note: For concentrations of nonprotein nitrogen in excess of 1000 mg per 100 ml, use 0.5 ml sample in Step 2 and multiply the values obtained from the calibration table for blood by 20.

1. Filter a small portion of thoroughly mixed urine.
2. In a 20 ml volumetric flask place 1 ml filtered urine and dilute to volume with ammonia-free distilled water (1).
3. In a 15 ml centrifuge tube place
   4 ml ammonia-free distilled water (1)
   1 ml diluted urine.
4. Add 5 ml 5% trichloracetic acid (2), drop by drop, with constant agitation of the tube.
5. Insert a clean rubber stopper in the tube, shake vigorously for 30 seconds and allow to stand at room temperature for 10 minutes.
6. Centrifuge at 1500 rpm for 3 minutes—if the supernatant fluid is not crystal clear, filter through a small Whatman #40 paper.
7. In a special dry digestion tube (sample) place
   1 ml clear supernatant centrifugate
   2.5 ml selenium-sulfuric acid digestion mixture "A" (3)
   1 solid glass bead.
8. Proceed exactly as outlined in the Test Procedure for blood, Steps 7–20 inclusive.
9. Measure the transmittancy of the sample against the reagent blank set at 100% transmission at a wave length of 415 mμ and multiply the value from calibration table for blood by 10 to obtain the concentration of nonprotein nitrogen in mg per 100 ml urine.

PROCEDURE FOR USE WITH LINEAR-SCALE PHOTOELECTRIC COLORIMETERS (SHEARD-SANFORD TYPE)

TEST PROCEDURE

Exactly as outlined; set the reagent blank at 100 and use a blue filter.

CALIBRATION PROCEDURE

Exactly as outlined—use a blue filter; plot the observed readings on semi-log graph paper—the points fall on a very slight curve passing through the concentration axis slightly to the right of the origin.

PROCEDURE FOR USE WITH LOGARITHMIC-SCALE PHOTOELECTRIC COLORIMETERS (KLETT-SUMMERSON TYPE)

TEST PROCEDURE

Exactly as outlined; set the reagent blank at 0 and use a blue filter.

CALIBRATION PROCEDURE

Exactly as outlined—use a blue filter; plot the observed readings on linear graph paper—the points fall on a very slight curve passing through the concentration axis slightly to the right of the origin.

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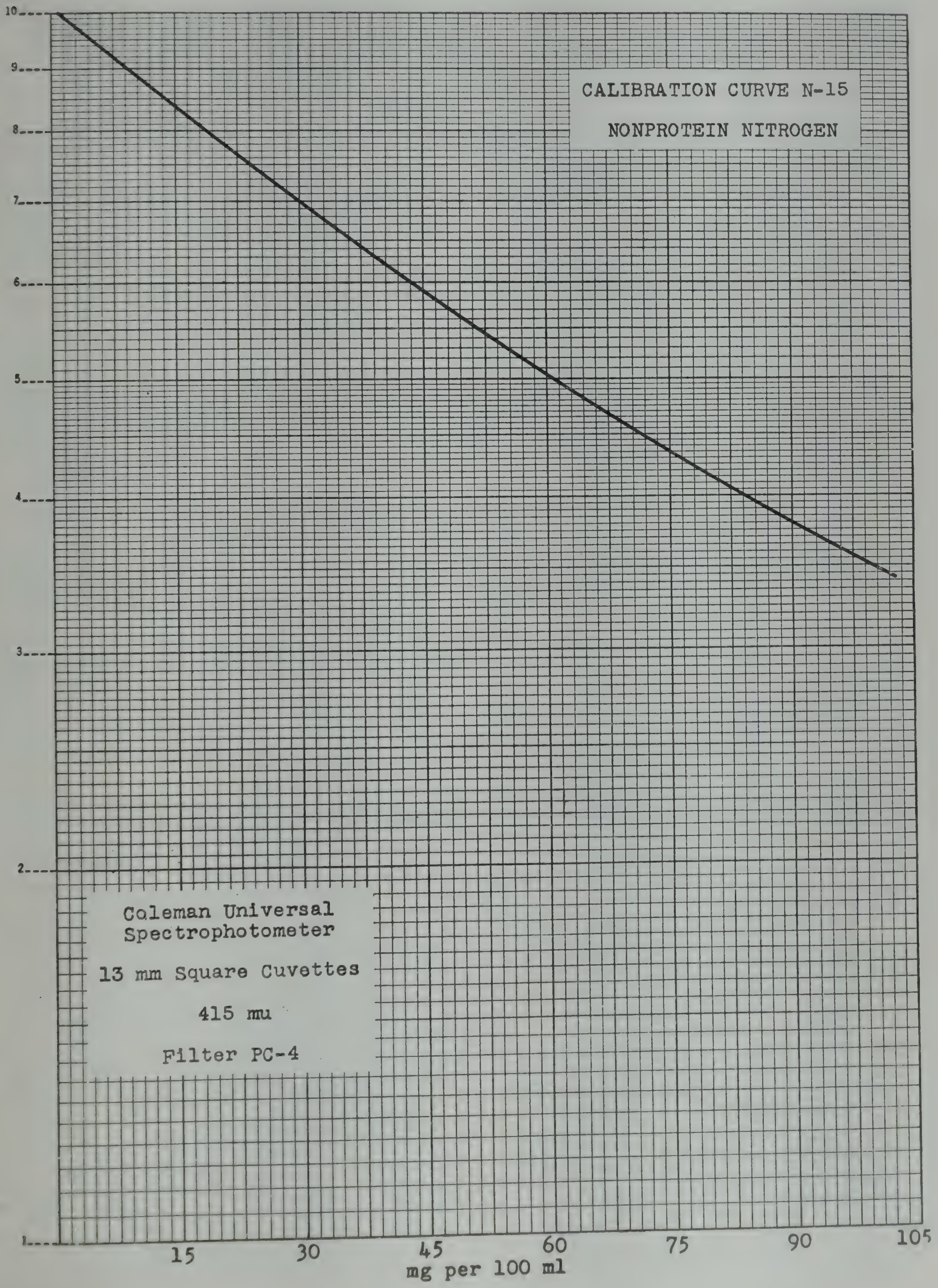
NOTES


CALIBRATION CURVE N-15
NONPROTEIN NITROGEN
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13 mm Square Cuvettes
415 mu
Filter PC-4
10
9
8
7
6
5
4
3
2
1
15
30
45
60
75
90
105
mg per 100 ml

Meter Reading	0	1	2	3	4	5	6	7	8	9
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10										
20										
30										
40										
50										
60										
70										
80										
90										

NITROGEN, TOTAL

A spectrophotometric adaptation based on the methods and modifications of Folin and Wu (1919), Myers (1921), Lauro (1931), Reis and Powers (1935), Campbell and Hanna (1937) and Hoffman and Osgood (1940)

PRINCIPLE

The suitably diluted sample is digested with a selenium-sulfuric acid mixture with the aid of hydrogen peroxide to convert nitrogenous constituents to ammonium sulfate in which the ammonia is determined by means of the reddish-orange color developed with alkaline potassium mercuric iodide (Nessler reaction).

DETERMINATION IN WHOLE BLOOD

SAMPLE

Blood withdrawn during post-absorptive state. Anticoagulant—potassium oxalate. Contamination by ammonia or its salts must be avoided.

TEST PROCEDURE

Special Apparatus

Micro pipettes accurately calibrated "to contain" 0.1 ml and 0.2 ml.

Fister digestion tubes—Pyrex; 25 × 200 mm; glass-stoppered; the 20 ml graduation must be accurately calibrated. These tubes prevent contamination during mixing as well as protect the hands from the action of the alkali. Folin-Wu tubes may be used provided that they are accurately calibrated at 20 ml.

Micro burner, Argand type.

Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.

Reagents

1. **Water, Distilled, Ammonia-Free.** In an all-glass Pyrex distillation apparatus place 1 gm sodium carbonate (Na_2CO_3—Baker & Adamson, A.C.S.) and 1 gm potassium permanganate ($KMnO_4$—Baker & Adamson, A.C.S.); add 500 ml distilled water; redistil, rejecting the first 100 ml portion and collecting only the next 300 ml. Use for the preparation of all reagents and standards and where indicated in the test procedure.
2. **Selenium-Sulfuric Acid Digestion Mixture "B" (Fister).** In a 1000 ml Florence flask place 450 ml ammonia-free distilled water (1); add slowly, while cooling the flask under running tap water, 33.6 ml concentrated sulfuric acid (H_2SO_4—Grasselli, A.C.S.); cool to room temperature; transfer to a 500 ml mixing cylinder; add 0.15 ml selenium oxychloride ($SeOCl_2$—J. T. Baker); dilute to volume with ammonia-free distilled water and mix thoroughly. Preserve in Pyrex glass-stoppered bottle. Note: This is not the same digestion mixture used in the nonprotein nitrogen and albumin determinations.
3. **Hydrogen Peroxide, 30%** (H_2O_2—Merck, Reagent Superoxol, Low-Nitrogen).
4. **Nessler Reagent (Folin-Wu).** In a 1000 ml mixing cylinder place 700 ml 2.5 N sodium hydroxide (a); add 150 ml double iodide solution (b); dilute to volume with ammonia-free distilled water (1) and mix; allow to stand for three days to permit sedimentation of the small amount of precipitate which forms. Preserve in ceresin-lined bottle. Stability indefinite. Note: The alkaline concentration of this reagent is 7%—11.0–11.5 ml must be required to neutralize 20 ml 1 N hydrochloric acid, using phenolphthalein as indicator.
   (a) Sodium hydroxide, volumetric 2.5 N; prepare by diluting slightly more than the required amount of concentrated carbonate-free sodium hydroxide solution (see Appendix); dilute 20 ml of this approximately 2.5 N solution to 100 ml; titrate against standard 0.5 N acid using phenolphthalein as indicator; adjust accordingly and retitrate—20 ml of 0.5 N acid require 20 ml of a 1:5 dilution of 2.5 N alkali. Preserve in ceresin-lined bottle.
   (b) Double iodide solution (Folin-Wu); in a 500 ml Florence flask place 150 gm granular potassium iodide (KI—Mallinckrodt, A.C.S.) and 100 gm resublimed iodine (I—Baker & Adamson, A.C.S.); add 100 ml ammonia-free distilled water (1) and shake until the iodine has completely dissolved; add 150 gm redistilled metallic mercury (Hg—Baker & Adamson, A.C.S.); shake the flask continuously and vigorously until the red color of the iodine solution has become visibly pale, though still red; immediately start cooling under running tap water, continuing vigorous shaking, until the reddish color has been replaced by the greenish-yellow color of the double iodide. If properly prepared, the solution is clear and there is a residue of shining metallic mercury; solutions showing a gray-green sludge are unsatisfactory. The whole operation should consume no more than 10–15 minutes. Since the reaction develops considerable heat, precautions should be taken to protect the hands during the shaking procedure. Decant the solution into a 2000 ml mixing cylinder, washing mercury and flask with liberal quantities of ammonia-free distilled water and decanting the washings into the cylinder. Dilute to volume with ammonia-free distilled water.

Technic

Note: For concentrations of nitrogen in excess of 5000 mg per 100 ml, dilute 0.1 ml blood in Step 1 with selenium-sulfuric acid mixture to 50 ml in a volumetric flask and multiply the values obtained from the calibration table by 2.

1. In a 25 ml volumetric flask place
   15 ml selenium-sulfuric acid digestion mixture "B" (2)
   0.1 ml blood from a micro pipette, thoroughly rinsing out the pipette by drawing in and expelling some of the mixture several times.
2. Dilute to volume with selenium-sulfuric acid digestion mixture "B" (2) and mix thoroughly.
3. In a special dry digestion tube (sample) place
   4 ml ammonia-free distilled water (1)
   0.2 ml diluted blood from a micro pipette, thoroughly rinsing out the pipette by drawing in and expelling some of the mixture several times
   1 solid glass bead.
4. Clamp tube in a vertical position on a support and apply gentle heat from an Argand micro burner in such a manner that the flame at no time touches the tube above the level of the liquid.

Note: Carry out Steps 4–10 inclusive in a fume hood.

5. After boiling has started gradually increase the heat and place burner in upright position so that the top is about $^3/_4$ inch below the bottom of the tube and the point of the flame directly under the tube—the bubbles should at no time rise above the middle of the tube.
6. Heat until white fumes of SO_3 fill about one-half of the tube—none of these fumes should escape.
7. Insert a small funnel into the mouth of the tube and continue gentle heating with reduced flame until the charred digest begins to clear.
8. Remove the flame and allow to cool for 90 seconds.
9. Remove the funnel and allow 1 drop 30% hydrogen peroxide (3) to fall directly on the digest.
10. Replace funnel and heat gently for 2 minutes making certain that any charred material which may have adhered to the walls of the tube has been washed down and decomposed—the digest should be perfectly clear—if not, add another drop of hydrogen peroxide in the same manner and heat again until clear.
11. Without removing the funnel, allow to cool for 2 minutes and then place the tube in a beaker half-full of ice-cold water.
12. In another special digestion tube (reagent blank) place 0.2 ml selenium-sulfuric acid digestion mixture "B" (2) and place in the cold water bath with the other tube.
13. After 10 minutes in the cold water bath, add about 8 ml ammonia-free distilled water (1) to each tube, rinsing off the funnel in the sample tube at the same time and mix, allowing the tubes to remain in the bath.
14. Without removing the tubes from the bath add 10 ml Nessler reagent (4), allowing the reagent to fall directly into the solution.
15. Remove the tubes from the bath, dilute to 20 ml with ammonia-free distilled water (1), insert the glass stoppers and mix by inversion.
16. Replace the tubes in the bath and allow to remain for 5 minutes.
17. Remove the tubes from the bath and allow to stand at room temperature (21° C.) for 10 minutes.
18. Measure the transmittancy of the sample against the reagent blank set at 100% transmission at a wave length of 415 mμ and obtain the concentration of nitrogen from the calibration table.

CALIBRATION PROCEDURE

Special Apparatus

Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.

Micro pipettes accurately calibrated "to contain" 0.1 ml and 0.2 ml.

Fister digestion tubes, glass-stoppered as described in the Test Procedure.

Micro burner, Argand type.

Reagents

1. **Water, Distilled, Ammonia-Free.** Same as for Test Procedure.
2. **Selenium-Sulfuric Acid Digestion Mixture "B" (Fister).** Same as for Test Procedure.
3. **Hydrogen Peroxide, 30%.** Same as for Test Procedure.
4. **Nessler Reagent (Folin-Wu).** Same as for Test Procedure.
5. **Nitrogen Standards**
   (a) Nitrogen stock standard (1 ml contains 10 mg nitrogen). Dry pyridine-free ammonium sulfate ($(NH_4)_2SO_4$—Reagent Chemicals, Special) at 110° C. for 1 hour; transfer 4.7166 gm to a 100 ml volumetric flask; add approximately 50 ml ammonia-free distilled water (1); shake until dissolved and dilute to volume with same. Preserve with a few drops of toluene. Stability indefinite.
   (b) Dilute standard (1 ml contains 2.5 mg nitrogen). In a 100 ml volumetric flask place 25 ml stock standard (5a) and dilute to volume with ammonia-free distilled water (1).
   (c) Calibration standards. In a series of five accurately calibrated 10 ml volumetric flasks place respectively 0, 1, 2, 3 and 4 ml of the dilute standard (5b) and dilute to volume with ammonia-free distilled water (1). These standards represent concentrations of nitrogen equivalent to 0, 1250, 2500, 3750 and 5000 mg per 100 ml sample

or 0, 1.25, 2.50, 3.75 and 5.0%. Prepare just before use.

Technic

1. Transfer 5 ml of each calibration standard (5c) to a correspondingly marked 25 ml volumetric flask.
2. Dilute to volume with ammonia-free distilled water (1) and mix thoroughly.
3. In each of a series of correspondingly marked special dry digestion tubes place
   - 4 ml ammonia-free distilled water (1)
   - 0.2 ml of the appropriate diluted calibration standard from Step 2, from a micro pipette, thoroughly rinsing out the pipette by drawing in and expelling some of the mixture several times
   - 0.2 ml selenium-sulfuric acid digestion mixture "B" (2)
   - 1 solid glass bead.
4. Treat each tube exactly as outlined in the Test Procedure, Steps 4–17 inclusive—the standards are run consecutively through Steps 4–11 inclusive, Step 12 is omitted (since this duplicates the zero concentration calibration standard) and then simultaneously thru Steps 13–17 inclusive.
5. Set the zero concentration standard at 100% transmission at a wave length of **415** mμ and record the transmittancy of the 1250, 2500, 3750 and 5000 mg per cent standards. The transmittancy of the 5000 mg standard should be approximately 30%.
6. Plot the observed values on semi-log graph paper —the points fall on a smooth curve which passes through the concentration axis slightly to the right of the origin (Calibration Curve N-16A).
7. Prepare a calibration table from the T-C values obtained from the plotted curve.

Method N-16b

DETERMINATION IN PLASMA OR SERUM

SAMPLE

Plasma or serum from blood withdrawn during post-absorptive state. Anticoagulant—potassium oxalate. There must be no trace of hemolysis.

TEST PROCEDURE

Special Apparatus

Micro pipettes accurately calibrated "to contain" 0.1 ml.

Ostwald-Folin pipettes accurately calibrated "to deliver" 0.5 ml.

Fister digestion tubes, glass-stoppered as described in the Test Procedure for whole blood.

Micro burner, Argand type.

Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.

Reagents

1. **Water, Distilled, Ammonia-Free.** Same as for whole blood.
2. **Selenium-Sulfuric Acid Digestion Mixture "B" (Fister).** Same as for whole blood.
3. **Hydrogen Peroxide, 30%.** Same as for whole blood.
4. **Nessler Reagent (Folin-Wu).** Same as for whole blood.

Technic

Note: For concentrations of nitrogen in excess of 2000 mg per 100 ml, dilute 0.1 ml sample in Step 1 with selenium-sulfuric acid mixture to 50 ml in a volumetric flask and multiply the values obtained from the calibration table for whole blood by 0.8; or multiply the values from the calibration table for plasma or serum by 2.

1. In a 25 ml volumetric flask place
   - 15 ml selenium-sulfuric acid digestion mixture "B" (2)
   - 0.1 ml sample from a micro pipette, thoroughly rinsing out the pipette by drawing in and expelling some of the mixture several times.
2. Dilute to volume with selenium-sulfuric acid digestion mixture "B" (2) and mix thoroughly.
3. In a special dry digestion tube (sample) place
   - 4 ml ammonia-free distilled water (1)
   - 0.5 ml diluted sample from an Ostwald-Folin pipette
   - 1 solid glass bead.
4. Proceed exactly as outlined in the Test Procedure for whole blood, Steps 4–17 inclusive, except that 0.5 ml selenium-sulfuric acid digestion mixture is used in the reagent blank in Step 12.
5. Measure the transmittancy of the sample against the reagent blank set at 100% transmission at a wave length of **415** mμ and multiply the value from the calibration table for whole blood by 0.4 to obtain the concentration of nitrogen in mg per 100 ml sample. If a calibration table has been prepared for total nitrogen in plasma or serum, the concentration is obtained directly.

CALIBRATION PROCEDURE

This calibration procedure need not be carried out. A calibration curve (Calibration Curve N-16B) may be constructed by substituting the values 500, 1000, 1500 and 2000 mg per 100 ml for the transmittancy readings recorded for the 1250, 2500, 3750 and 5000 mg per 100 ml concentrations in the calibration curve for whole blood.

Method N-16c

DETERMINATION IN URINE

SAMPLE

Aliquot of a 24-hour specimen. Keep cool during collection.

TEST PROCEDURE

Special Apparatus

As listed under the Test Procedure for whole blood.

Reagents

1. **Water, Distilled, Ammonia-Free.** Same as for whole blood.
2. **Selenium-Sulfuric Acid Digestion Mixture "B" (Fister).** Same as for whole blood.
3. **Hydrogen Peroxide, 30%.** Same as for whole blood.
4. **Nessler Reagent (Folin-Wu).** Same as for whole blood.

Technic

Note: For concentrations of nitrogen in excess of 2000 mg per 100 ml, dilute 0.1 ml urine in Step 2 with selenium-sulfuric acid mixture to 50 ml in a volumetric flask and multiply the values obtained from the calibration table for whole blood by 0.8.

1. Filter a small portion of thoroughly mixed urine.
2. In a 25 ml volumetric flask place
   15 ml selenium-sulfuric acid digestion mixture "B" (2)
   0.1 ml filtered urine from a micro pipette, thoroughly rinsing out the pipette by drawing in and expelling some of the mixture several times.
3. Dilute to volume with selenium-sulfuric acid digestion mixture "B" (2) and mix thoroughly.
4. In a special dry digestion tube (sample) place
   4 ml ammonia-free distilled water (1)
   0.5 ml diluted urine
   1 solid glass bead.
5. Proceed exactly as outlined in the Test Procedure for whole blood, Steps 4–17 inclusive, except that 0.5 ml selenium-sulfuric acid digestion mixture is used in the reagent blank in Step 12.
6. Measure the transmittancy of the sample against the reagent blank set at 100% transmission at a wave length of 415 mμ and multiply the value from the calibration table for whole blood by 0.4 to obtain the concentration of nitrogen in mg per 100 ml urine.

PROCEDURE FOR USE WITH LINEAR-SCALE PHOTOELECTRIC COLORIMETERS (SHEARD-SANFORD TYPE)

TEST PROCEDURE

Exactly as outlined; set the reagent blank at 100 and use a blue filter.

CALIBRATION PROCEDURE

Exactly as outlined—use a blue filter; plot the observed readings on semi-log graph paper—the points fall on a very slight curve passing through the concentration axis slightly to the right of the origin.

PROCEDURE FOR USE WITH LOGARITHMIC-SCALE PHOTOELECTRIC COLORIMETERS (KLETT-SUMMERSON TYPE)

TEST PROCEDURE

Exactly as outlined; set the reagent blank at 0 and use a blue filter.

CALIBRATION PROCEDURE

Exactly as outlined—use a blue filter; plot the observed readings on linear graph paper—the points fall on a very slight curve passing through the concentration axis slightly to the right of the origin.

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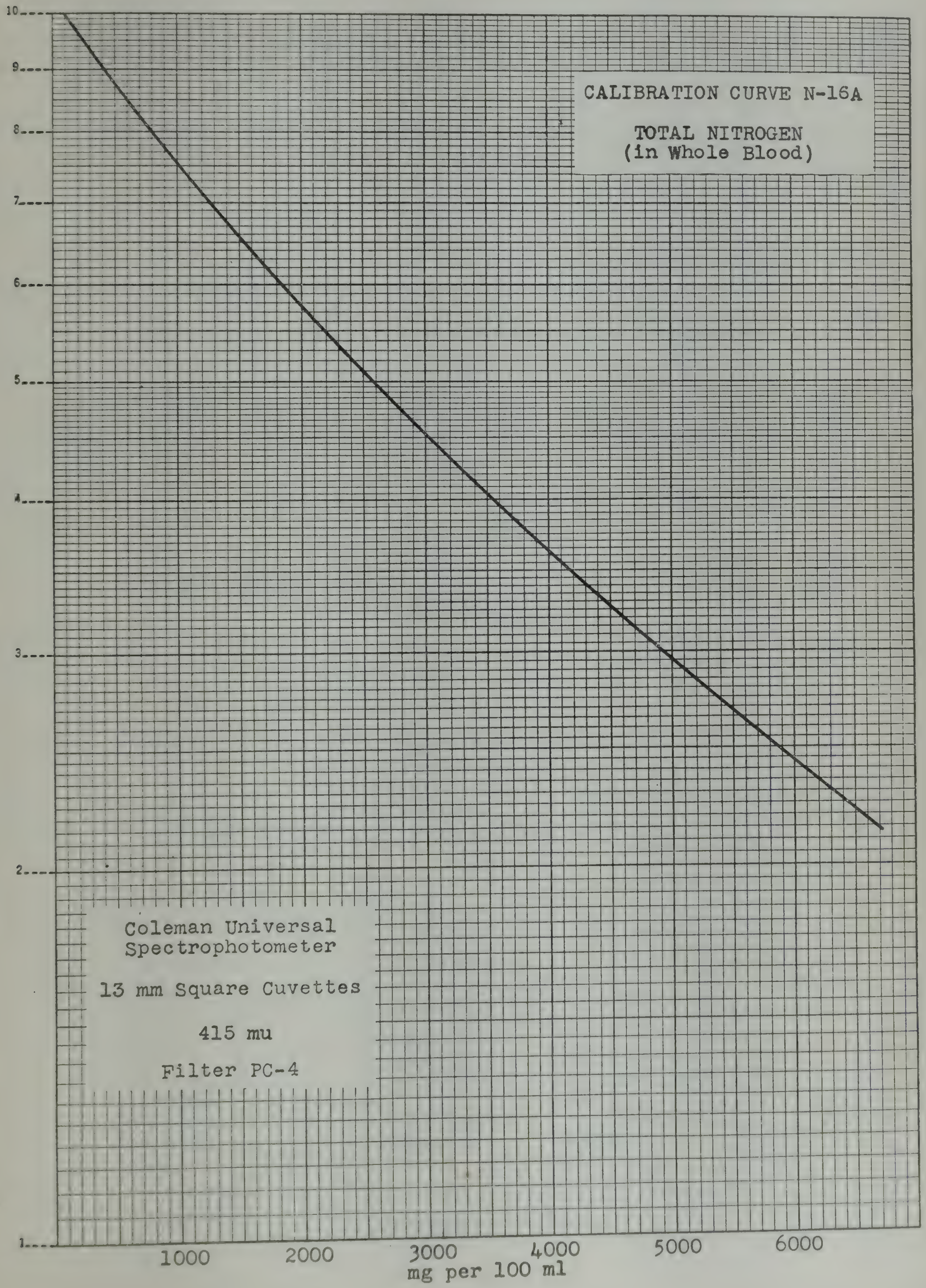
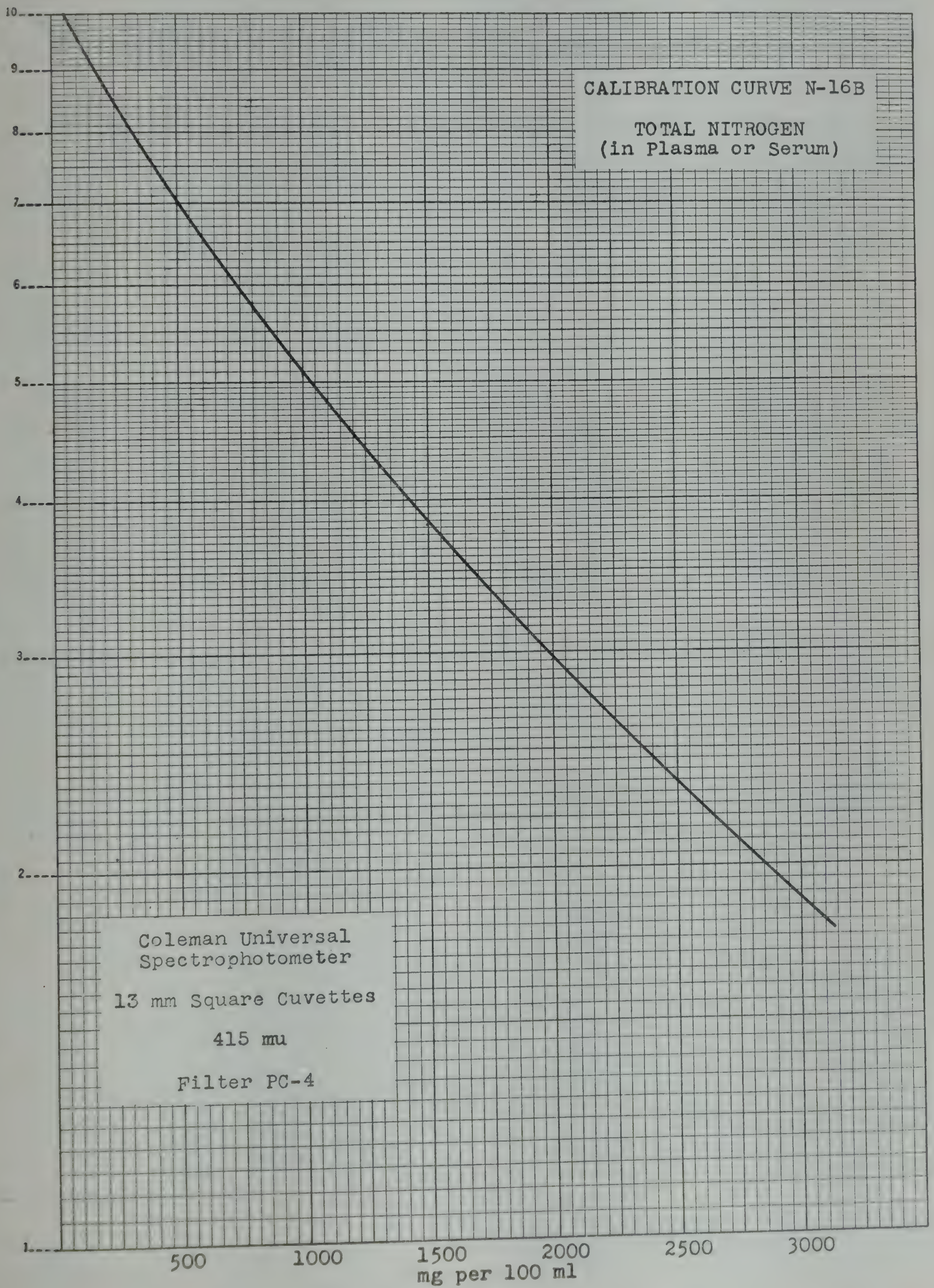

CALIBRATION CURVE N-16A
TOTAL NITROGEN
(in Whole Blood)
Coleman Universal
Spectrophotometer
13 mm Square Cuvettes
415 mu
Filter PC-4
10
9
8
7
6
5
4
3
2
1
1000
2000
3000
4000
5000
6000
mg per 100 ml

Meter Reading	0	1	2	3	4	5	6	7	8	9
0										
10										
20										
30										
40										
50										
60										
70										
80										
90										


CALIBRATION CURVE N-16B
TOTAL NITROGEN
(in Plasma or Serum)
Coleman Universal Spectrophotometer
13 mm Square Cuvettes
415 mu
Filter PC-4
10
9
8
7
6
5
4
3
2
1
500
1000
1500
2000
2500
3000
mg per 100 ml

Meter Reading	0	1	2	3	4	5	6	7	8	9
0										
10										
20										
30										
40										
50										
60										
70										
80										
90										

OXYGEN CAPACITY

A spectrophotometric adaptation based on the methods and modifications of Exton, Schattner and Rose (1941) and Exton, Schattner, Korman and Rose (1945)

PRINCIPLE

When whole blood, saturated with atmospheric oxygen, is treated with ferrous sulfate in a medium rendered slightly alkaline by sodium tetraborate, the available oxygen converts the ferrous hydroxide to ferric hydroxide which reacts with sulfosalicylic acid to produce a red color, the density of which is proportional to the amount of ferric iron present and indirectly to the available oxygen in the sample. The sulfosalicylic acid simultaneously precipitates the proteins and halts the oxidation process.

DETERMINATION IN WHOLE BLOOD

SAMPLE

Blood withdrawn at any time of the day. Heparin must be used as an anticoagulant—fluorides or oxalates cannot be used. Analyses should be made as soon as possible after withdrawal of the blood. For oxygen capacity determinations, collection under oil is not essential but samples so collected may also be used for the determination of oxygen content (Method O-12). In addition, the layer of oil prevents evaporation and contamination by dust.

COLLECTION: (a) Capillary blood: Connect a collecting cup (Fig. O-10a) to a prepared centrifuge tube (Fig. O-10b) and fill tube and cup with mineral oil (Reagent #2) to about one inch below the edge of the cup. Puncture the finger, wipe off the first drop of blood, and quickly submerge the finger tip under the oil in the cup; as soon as the volume of blood reaches or exceeds the 0.5 ml mark on the tube, remove the finger from the cup. Mix blood and anticoagulant thoroughly by stirring with a thin glass rod; carefully pour the oil out of the cup and the upper third of the centrifuge tube; detach the cup and place the centrifuge tube in the refrigerator.

(b) Venous blood: To a small prepared sample vial add sufficient mineral oil (Reagent #2) to form a layer about 1 cm in height. Carefully withdraw about 6 ml venous blood, using a thoroughly clean, dry syringe and needle and avoiding the inclusion of any air bubbles. Immediately place the point of the needle under the layer of mineral oil in the sample vial and *slowly* expel 4 ml of the blood, retaining about 2 ml in the syringe—the retained blood may be used for other purposes or discarded. Mix by gentle agitation avoiding contamination by atmospheric oxygen.

TEST PROCEDURE

Special Apparatus

A single determination requires the following:

For capillary blood

Collection cup with ground glass joint (Fig. O-10a).

Centrifuge tube, 5 ml, graduated at 0.5 ml, with ground glass joint to fit the collection cup (Fig. O-10b). Prepare this tube before use by adding one small drop freshly diluted 0.3% heparin (Reagent #1), distribute evenly over the bottom of the tube and allow to stand until dry.

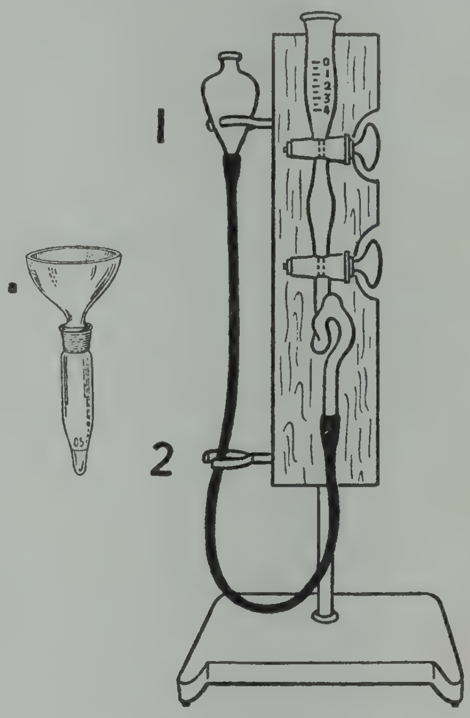


Fig. O-10.

For venous blood

Sample vial, small, wide-mouth. Prepare this vial before use by adding 0.1 ml freshly diluted 0.3% heparin (Reagent #1), distribute evenly over the bottom of the vial and allow to stand until dry.

For both capillary and venous blood

Capillary pipettes with rubber bulbs.

Ostwald-Van Slyke pipettes, accurately calibrated "to deliver" 0.1 ml.

Reaction chamber, the upper end of which is connected to a graduated funnel cup by means of a stopcock and the lower end, by means of another stopcock, to a rubber tube with mercury and mercury-levelling bulb (Fig. O-10)—two assemblies are required, one for the sample and the other for the blank.

Graduated centrifuge tubes—25 ml, the 20 ml graduation must be accurately calibrated.

Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.

Note: It is essential that all glassware be absolutely clean. Wash with dichromate-sulfuric acid solution (see Appendix). Be especially careful that there is no blood or protein residue in the reaction chamber or fat or grease on any of the glassware. The cleaning solutions must not introduce iron as a contaminant.

Reagents

1. **Heparin, 0.3% Aqueous.** Just before use add 0.3 ml 1% heparin (a) to 0.7 ml distilled water and mix.
   (a) Heparin, 1% aqueous; dissolve 100 mg heparin (Connaught Laboratories) in 10 ml distilled water. Preserve in a vaccine bottle in refrigerator.
2. **Mineral Oil** (Marcol GX—Standard Oil Co. of U.S.).
3. **Water, Distilled, Iron-Free.** Redistil water in an all-glass Pyrex distillation apparatus until iron-free. To test for the presence of iron, treat 10 ml with 1 ml concentrated hydrochloric acid, 0.1 ml concentrated nitric acid, 4 ml 3 N potassium thiocyanate and 2 ml iso-amyl alcohol; shake thoroughly and allow the alcohol layer to separate; a colorless alcohol layer indicates the absence of iron. Use for the preparation of reagents and standards where indicated and throughout the test procedure.
4. **Sodium Tetraborate, 2% Aqueous.** Dissolve 2 gm sodium tetraborate ($Na_2B_4O_7 \cdot 10H_2O$—Baker & Adamson, A.C.S.) in iron-free distilled water (3) and dilute to 100 ml.
5. **Ferrous Sulfate, 5% Aqueous.** In a 50 ml beaker place 500 mg crystalline ferrous sulfate ($FeSO_4 \cdot 7H_2O$—Baker & Adamson, Reagent); add 10 ml distilled water and a small amount of celite (Reagent #7); rapidly bring to a boil and boil gently for 1 minute; immediately transfer to a 15 ml centrifuge tube, cover with a 1 cm layer of mineral oil (2) and centrifuge—the reagent under the layer of oil is free of ferric iron. Prepare fresh daily. Note: Contamination with atmospheric oxygen must be avoided. When withdrawing the reagent for use, place the tip of the pipette near the center of the column of fluid—away from the oil-water interphase and from the bottom of the tube.
6. **Sulfosalicylic Acid, 30% Aqueous.** Dissolve 30 gm crystalline sulfosalicylic acid ($SO_3H \cdot C_6H_3 \cdot OH \cdot COOH$—Merck, Reagent) in iron-free distilled water (3) and dilute to 100 ml. Only pure white crystals should be used and solutions showing a pinkish tinge should be discarded. Allow to stand for 24 hours until sedimentation is complete; filter through a Schleicher and Schuell #576 paper. Preserve in resistant-glass bottle well protected from dust.
7. **Celite** (Johns-Manville Co.).

Technic

Note: A blank determination is run at the same time and in the same manner as the analytical procedure described in the following steps except that the addition of blood in Step 6 is omitted.

1. Set up the apparatus and place the mercury-levelling bulb in position 1 as illustrated (Fig. O-10); with stopcocks open, add sufficient mercury through the levelling bulb so that the level of mercury is at the 1 ml mark on the funnel cup above the reaction chamber; adjust position 2 so that the mercury level is in the taper just above the lower stopcock.
2. Place slightly more than 4 ml 2% sodium tetraborate (4) in the funnel cup—make certain that minute air bubbles are absent by drawing some of the solution into the reaction chamber, turning the stopcock and forcing the mercury up and down.
3. Bring the mercury level just to the top of the bore in the upper stopcock and adjust the tetraborate solution exactly to the zero mark; allow about 0.2 ml of the tetraborate solution to come below the upper stopcock; close the lower stopcock but leave the upper open.
4. By means of a capillary pipette with rubber bulb withdraw about 0.2 ml blood from the sample container and carefully remove adherent oil with a tissue; transfer to a clean dry sample vial which does not contain heparin.
5. Roll the blood over the bottom and the sides of the vial in such a manner as to avoid the inclusion of air bubbles—this should be carried out fairly rapidly so as to prevent concentration by evaporation.
6. Transfer 0.1 ml of the oxygen-saturated blood to the funnel cup by means of an Ostwald-Van Slyke pipette, place the pipette in the tetraborate solution so that the tip is just above the stopcock and allow the blood to flow slowly through the bore of the stopcock and settle on the surface of the mercury—do not allow the blood to back-up and rise above the tip of the pipette.
7. Immediately add 0.1 ml 5% ferrous sulfate (5) by means of an Ostwald-Van Slyke pipette (wiped free of adherent oil)—introduce the pipette into the tetraborate solution in the manner described in Step 6 and allow the reagent to flow through the bore of the stopcock at moderate speed—the ferrous sulfate must at no time come in contact with atmospheric oxygen. Note: When the ferrous sulfate flows into the tetraborate solution a fine bluish-green precipitate is produced and when the pipette is withdrawn some of the precipitate may be carried upward by the current—this is of no consequence.
8. Immediately draw the solution in the cup into the reaction chamber until the level in the cup is at exactly the 1.5 ml mark—this flushes the ferrous hydroxide suspension into the chamber before any contaminated particles which have risen to the surface have settled.
9. Close both stopcocks, aspirate off the excess tetraborate solution in the cup and mix the contents

of the reaction chamber by gentle shaking for 15 seconds.

10. Allow to stand for 2 minutes.
11. Rinse the cup three times with 5 ml portions iron-free distilled water (3) and discard the washings.
12. Place exactly 2 ml 30% sulfosalicylic acid (6) in the cup, raise the levelling bulb, open the upper stopcock and then the lower and allow all the fluid in the reaction chamber plus a little of the mercury to flow slowly up into the acid in the cup, stirring the mixture in the cup constantly with a thin glass rod—air bubbles must not be introduced when adding the acid.
13. Transfer the colored fluid in the cup to a 25 ml graduated centrifuge tube by means of a capillary pipette fitted with a rubber bulb.
14. Rinse the funnel cup and stirring rod with successive small portions iron-free distilled water (3) and transfer the washings to the centrifuge tube until the level of the fluid in the tube is at exactly 20 ml.
15. Add about 1 mg celite (7) and shake thoroughly.
16. Allow to stand for 5 minutes.
17. Centrifuge at 1800 rpm for 3 minutes (or filter through iron-free paper).
18. In a cuvette place the crystal-clear sample centrifugate (or filtrate).
19. In another cuvette place the crystal-clear reagent blank centrifugate (or filtrate).
20. Measure the transmittancy of the sample against the reagent blank set at 100% transmission at a wave length of **510 mμ** and obtain the apparent oxygen capacity in volumes per cent from the calibration table. Calculate the true oxygen capacity as follows.

Calculation

Apparent oxygen capacity in volumes % from calibration table − 0.5 volume % = True oxygen capacity in volumes %

CALIBRATION PROCEDURE

Special Apparatus

Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.

Reagents

3. **Water, Distilled, Iron-Free.** Same as for Test Procedure.
6. **Sulfosalicylic Acid, 30% Aqueous.** Same as for Test Procedure.
8. **Oxygen Capacity Standards**
   (a) Oxygen capacity stock standard (1 ml contains 2.592 mg ferric ammonium sulfate). In a 100 ml volumetric flask place 259.2 mg violet ferric ammonium sulfate crystals ($Fe_2(SO_4)_3(NH_4)_2SO_4 \cdot 24H_2O$—Baker & Adamson, A.C.S.); add 50 ml iron-free distilled water (3); shake until dissolved; add 4 ml 30% sulfosalicylic acid (6) and mix; dilute to volume with iron-free distilled water.
   (b) Calibration standards. In a series of six accurately calibrated 100 ml volumetric flasks place respectively 0, 1, 2, 3, 4 and 5 ml of the stock standard (8a); add 10 ml 30% sulfosalicylic acid (6) to each flask and mix; dilute to volume with iron-free distilled water (3). These standards represent available oxygen concentrations of 0, 6, 12, 18, 24 and 30 volumes per cent. Prepare just before use.

Technic

1. Transfer a suitable portion of each calibration standard (8b) to a correspondingly marked cuvette.
2. Set the zero concentration standard at 100% transmission at a wave length of **510 mμ** and record the transmittancy of the 6, 12, 18, 24 and 30 per cent standards. The transmittancy of the 30% standard should be approximately 25.5%.
3. Plot the observed values on semi-log graph paper—the points fall on a straight line which passes through the origin (Calibration Curve O-10).
4. Prepare a calibration table from the T-C values obtained from the plotted curve.

CONVERSION FACTOR

$$\frac{\text{Oxygen capacity in volumes per cent}}{1.36} = \text{Hemoglobin in gm per 100 ml}$$

PROCEDURE FOR USE WITH LINEAR-SCALE PHOTOELECTRIC COLORIMETERS (SHEARD-SANFORD TYPE)

TEST PROCEDURE

Exactly as outlined; set the reagent blank at 100 and use a green filter.

CALIBRATION PROCEDURE

Exactly as outlined—use a green filter; plot the observed readings on semi-log graph paper—the points fall on a straight line passing through the origin.

PROCEDURE FOR USE WITH LOGARITHMIC-SCALE PHOTOELECTRIC COLORIMETERS (KLETT-SUMMERSON TYPE)

TEST PROCEDURE

Exactly as outlined; set the reagent blank at 0 and use a green filter.

CALIBRATION PROCEDURE

Exactly as outlined—use a green filter; plot the observed readings on linear graph paper—the points fall on a straight line passing through the origin.

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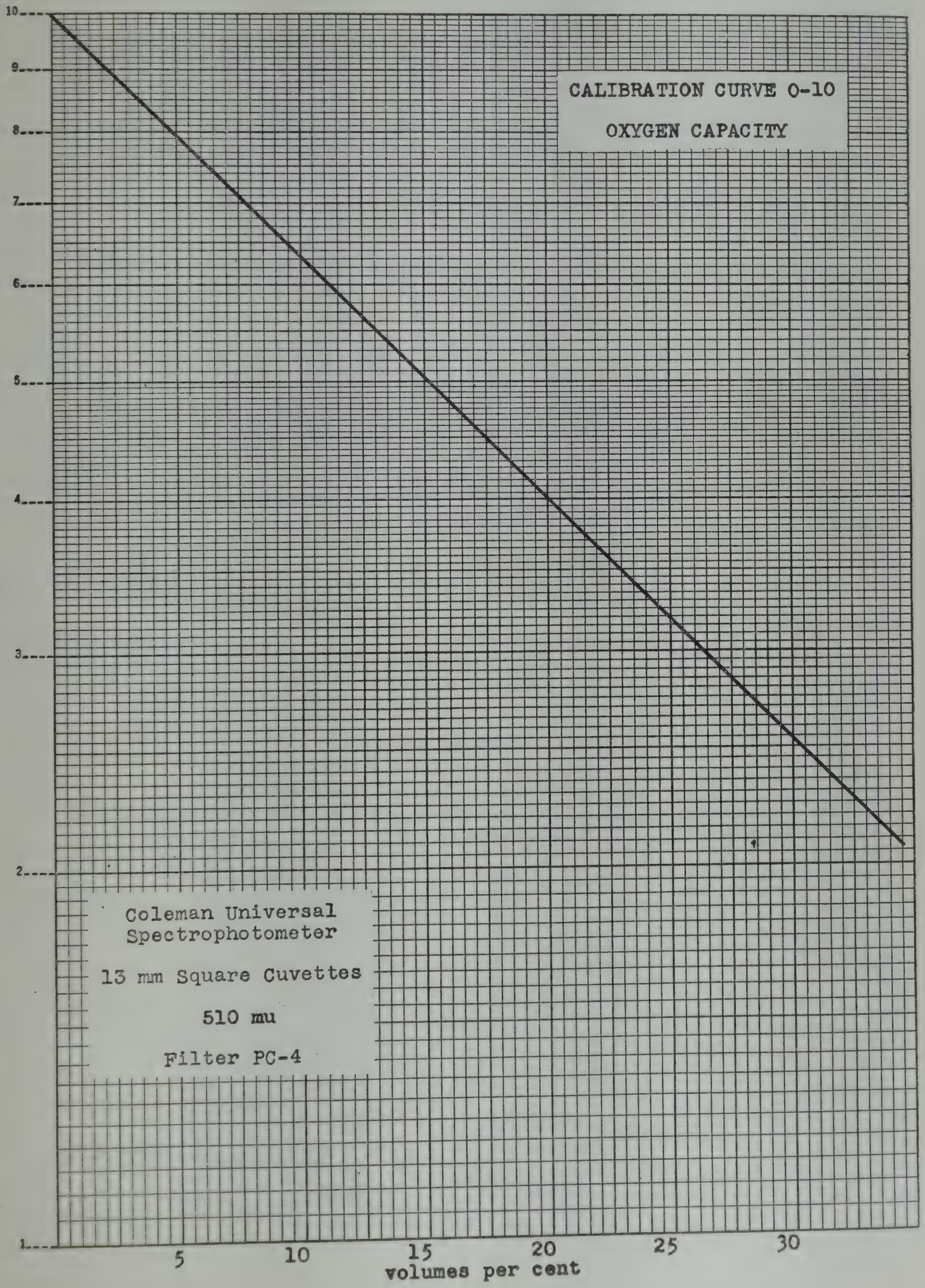
NOTES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OXYGEN CAPACITY
Coleman Universal Spectrophotometer
13 mm Square Cuvettes
510 mu
Filter PC-4
10
9
8
7
6
5
4
3
2
1
5
10
15
20
25
30
volumes per cent

Meter Reading	0	1	2	3	4	5	6	7	8	9
0										
10										
20										
30										
40										
50										
60										
70										
80										
90										

OXYGEN CAPACITY
(Indirect Iron Method)

Oxygen capacity may be indirectly estimated as follows by determining the iron content in whole blood—

1. Determine total iron in mg per 100 ml whole blood as outlined in Methods I-27 or I-28.
2. Calculation—

Total iron in mg per 100 ml × 0.4 = Oxygen capacity in volumes %

NOTES

NOTES

OXYGEN CONTENT

A spectrophotometric adaptation based on the methods and modifications of Exton, Schattner and Rose (1941) and Exton, Schattner, Korman and Rose (1945)

PRINCIPLE

Whole blood, without preliminary saturation with atmospheric oxygen, is analyzed as described in the method for oxygen capacity (Method O-10), the same analytical principles being involved.

DETERMINATION IN WHOLE BLOOD

SAMPLE

Blood withdrawn at any time of the day. Heparin must be used as an anticoagulant—fluorides or oxalates cannot be used. Analyses should be made as soon as possible after withdrawal of the blood. For oxygen content determinations, collection under oil is essential.

Collection

(a) Capillary blood: Connect a collecting cup (Fig. O-10a) to a prepared centrifuge tube (Fig. O-10b) and fill the tube and cup with mineral oil (Reagent #2) to about one inch below the edge of the cup. Puncture the finger, wipe off the first drop of blood, and quickly submerge the finger tip under the oil in the cup; as soon as the volume of blood reaches or exceeds the 0.5 ml mark on the tube, remove the finger from the cup. Mix blood and anticoagulant thoroughly by stirring with a thin glass rod; carefully pour the oil out of the cup and the upper third of the centrifuge tube; detach the cup and place the centrifuge tube in the refrigerator.

(b) Venous blood: To a small prepared sample vial add sufficient mineral oil (Reagent #2) to form a layer about 1 cm in height. Carefully withdraw about 6 ml venous blood, using a thoroughly clean, dry syringe and needle and avoiding the inclusion of any air bubbles. Immediately place the point of the needle under the layer of mineral oil in the sample vial and *slowly* expel 4 ml of the blood, retaining about 2 ml in the syringe—the retained blood may be used for other purposes or discarded. Mix by gentle agitation avoiding contamination by atmospheric oxygen.

TEST PROCEDURE

Special Apparatus

A single determination requires the following:

For capillary blood
  Collection cup with ground glass joint (Fig. O-10a).
  Centrifuge tube, 5 ml, graduated at 0.5 ml, with ground glass joint to fit the collection cup (Fig. O-10b). Prepare this tube before use by adding one small drop freshly diluted 0.3% heparin (Reagent #1), distribute evenly over the bottom of the tube and allow to stand until dry.
For venous blood
  Sample vial, small, wide-mouth. Prepare this vial before use by adding 0.1 ml freshly diluted 0.3% heparin (Reagent #1), distribute evenly over the bottom of the vial and allow to stand until dry.
For both capillary and venous blood
  Capillary pipettes with rubber bulbs.
  Ostwald-Van Slyke pipettes, accurately calibrated "to deliver" 0.1 ml.
  Reaction chamber, the upper end of which is connected to a graduated funnel cup by means of a stopcock and the lower end, by means of another stopcock, to a rubber tube with mercury and mercury-levelling bulb (Fig. O-10f)—two assemblies are required, one for the sample and the other for the blank.
  Graduated centrifuge tubes—25 ml, the 20 ml graduation must be accurately calibrated.
  Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.

Note: It is essential that all glassware be absolutely clean. Wash with dichromate-sulfuric acid solution (see Appendix). Be especially careful that there is no blood or protein residue in the reaction chamber or fat or grease on any of the glassware. The cleaning solutions must not introduce iron as a contaminant.

Reagents

1. **Heparin, 0.3% Aqueous.** Just before use add 0.3 ml 1% heparin (a) to 0.7 ml distilled water and mix.
   (a) Heparin, 1% aqueous; dissolve 100 mg heparin (Connaught Laboratories) in 10 ml distilled water. Preserve in a vaccine bottle in refrigerator.
2. **Mineral Oil** (Marcol GX—Standard Oil Co. of U.S.).
3. **Water, Distilled, Iron-Free.** Redistil distilled water in an all-glass Pyrex distillation apparatus until iron-free. To test for the presence of iron, treat 10 ml with 1 ml concentrated hydrochloric acid, 0.1 ml concentrated nitric acid, 4 ml 3 N potassium thiocyanate and 2 ml iso-amyl alcohol; shake thoroughly and allow the alcohol layer to separate; a colorless alcohol layer indicates the absence of iron. Use for the preparation of reagents and standards where indicated and throughout the test procedure.
4. **Sodium Tetraborate, 2% Aqueous** Dissolve 2 gm sodium tetraborate ($Na_2B_4O_7 \cdot 10H_2O$—

Baker & Adamson, A.C.S.) in iron-free distilled water (3) and dilute to 100 ml.

5. **Ferrous Sulfate, 5% Aqueous.** In a 50 ml beaker place 500 mg crystalline ferrous sulfate ($FeSO_4 \cdot 7H_2O$—Baker & Adamson, Reagent); add 10 ml distilled water and a small amount of celite (Reagent #7); rapidly bring to a boil and boil gently for 1 minute; immediately transfer to a 15 ml centrifuge tube, cover with a 1 cm layer of mineral oil (2) and centrifuge—the reagent under the layer of oil is free of ferric iron. Prepare fresh daily. Note: Contamination with atmospheric oxygen must be avoided. When withdrawing the reagent for use, place the tip of the pipette near the center of the column of fluid—away from the oil-water interphase and from the bottom of the tube.
6. **Sulfosalicylic Acid, 30% Aqueous.** Dissolve 30 gm crystalline sulfosalicylic acid ($SO_3H \cdot C_6H_3 \cdot OH \cdot COOH$—Merck, Reagent) in iron-free distilled water (3) and dilute to 100 ml. Only pure white crystals should be used and solutions showing a pinkish tinge should be discarded. Allow to stand for 24 hours until sedimentation is complete; filter thru a Schleicher and Schuell #576 paper. Preserve in resistant-glass bottle well protected from dust.
7. **Celite** (Johns-Manville Co.).

Technic

Note: A blank determination is run at the same time and in the same manner as the analytical procedure described in the following steps except that the addition of blood in Step 5 is omitted.

1. Set up the apparatus and place the mercury-levelling bulb in position 1 as illustrated (Fig. O-10); with stopcocks open, add sufficient mercury through the levelling bulb so that the level of mercury is at the 1 ml mark on the funnel cup above the reaction chamber; adjust position 2 so that the mercury level is in the taper just above the lower stopcock.
2. Place slightly more than 4 ml 2% sodium tetraborate (4) in the funnel cup—make certain that minute air bubbles are absent by drawing some of the solution into the reaction chamber, turning the stopcock and forcing the mercury up and down.
3. Bring the mercury level just to the top of the bore in the upper stopcock and adjust the tetraborate solution exactly to the zero mark; allow about 0.2 ml of the tetraborate solution to come below the upper stopcock; close the lower stopcock but leave the upper open.
4. By means of an Ostwald-Van Slyke pipette, withdraw slightly more than the required amount of blood from the sample container and carefully remove adherent oil with a tissue. Note: When withdrawing the blood, place the tip of the pipette near the center of the column of blood—away from the oil-blood interphase and from the bottom of the tube.
5. Adjust the volume and transfer exactly 0.1 ml to the funnel cup—place the pipette in the tetraborate solution so that the tip is just above the stopcock and allow the blood to flow slowly through the bore of the stopcock and settle on the surface of the mercury—do not allow the blood to back-up and rise above the tip of the pipette.
6. Immediately add 0.1 ml 5% ferrous sulfate (5) by means of an Ostwald-Van Slyke pipette (wiped free of adherent oil)—introduce the pipette into the tetraborate solution in the manner described in Step 5 and allow the reagent to flow through the bore of the stopcock at moderate speed—the ferrous sulfate must at no time come in contact with atmospheric oxygen. Note: When the ferrous sulfate flows into the tetraborate solution a fine bluish-green precipitate is produced and when the pipette is withdrawn some of the precipitate may be carried upward by the current—this is of no consequence.
7. Immediately draw the solution in the cup into the reaction chamber until the level in the cup is at exactly the 1.5 ml mark—this flushes the ferrous hydroxide suspension into the chamber before any contaminated particles which have risen to the surface have settled.
8. Close both stopcocks, aspirate off the excess tetraborate solution in the cup and mix the contents of the reaction chamber by gentle shaking for 15 seconds.
9. Allow to stand for 2 minutes.
10. Rinse the cup three times with 5 ml portions iron-free distilled water (3) and discard the washings.
11. Place exactly 2 ml 30% sulfosalicylic acid (6) in the cup, raise the levelling bulb, open the upper stopcock and then the lower and allow all the fluid in the reaction chamber plus a little of the mercury to flow slowly up into the acid in the cup, stirring the mixture in the cup constantly with a thin glass-rod—air bubbles must not be introduced when adding the acid.
12. Transfer the colored fluid in the cup to a 25 ml graduated centrifuge tube by means of a capillary pipette fitted with a rubber bulb.
13. Rinse the funnel cup and stirring rod with successive small portions iron-free distilled water (3) and transfer the washings to the centrifuge tube until the level of the fluid in the tube is at exactly 20 ml.
14. Add about 1 mg celite (7) and shake thoroughly.
15. Allow to stand for 5 minutes.
16. Centrifuge at 1800 rpm for 3 minutes (or filter through iron-free paper).
17. In a cuvette place the crystal-clear sample centrifugate (or filtrate).
18. In another cuvette place the crystal-clear reagent blank centrifugate (or filtrate).
19. Measure the transmittancy of the sample against the reagent blank set at 100% transmission at a

wave length of 510 mμ and obtain the concentration of oxygen directly from the calibration table for oxygen capacity (Method O-10).

CALIBRATION PROCEDURE

This calibration procedure need not be carried out. The values for oxygen are obtained directly from the calibration table for oxygen capacity (Method O-10).

PROCEDURE FOR USE WITH LINEAR-SCALE PHOTOELECTRIC COLORIMETERS (SHEARD-SANFORD TYPE)

TEST PROCEDURE

Exactly as outlined; set the reagent blank at 100 and use a green filter.

CALIBRATION PROCEDURE

Need not be performed; values obtained directly from calibration table for oxygen capacity.

PROCEDURE FOR USE WITH LOGARITHMIC-SCALE PHOTOELECTRIC COLORIMETERS (KLETT-SUMMERSON TYPE)

TEST PROCEDURE

Exactly as outlined; set the reagent blank at 0 and use a green filter.

CALIBRATION PROCEDURE

Need not be performed; values obtained directly from calibration table for oxygen capacity.

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NOTES

NOTES

PHENOL, FREE

A spectrophotometric adaptation based on the methods and modifications of Moir (1922), Theis and Benedict (1924), Schmidt, Schmulovitz, Szczpinski and Wylie (1937) and Schmidt (1942; 1943)

PRINCIPLE

Free phenol, in a protein-free filtrate, is extracted with ether at a definite pH and the extract, in alcohol-ether solution, treated with diazotized p-nitroaniline to produce a red color (Moir reaction). Since the color produced by normal amounts of phenol is too pale to permit accurate measurement in the range covered, a known amount of phenol is added to the extract of the unknown to enhance the color intensity. Due to the fact that recovery from the blood is approximately only 75%, a correction factor must be applied.

DETERMINATION IN WHOLE BLOOD

SAMPLE

Blood withdrawn during post-absorptive state. Anticoagulant—potassium oxalate. The determination cannot be carried out during the administration of sulfonamides.

TEST PROCEDURE

Special Apparatus

Centrifuge tubes, 100 ml.

Schmidt-Wylie continuous extraction assembly (Fig. P-10) which consists of the following:
- Condenser, West type, with ground glass joints at top and bottom—a solid glass stopper is fitted loosely in the upper joint (Fig. P-10a).
- Coaxial extraction tube (Fig. P-10b).
- Glass tubing, 6 mm o.d., funnel shaped at the top and drawn to a small opening at the bottom (Fig. P-10c).
- Receiver, small, 13 × 100 mm, graduated at the 3 ml mark (Fig. P-10d).
- Receiver, large, 18 × 150 mm, graduated at 3 ml intervals.

Note: The use of apparatus with glass joints is essential—corks or rubber stoppers and connections cannot be used.

Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.

Reagents

1. **Sodium Tungstate, 10% Aqueous.** Dissolve 10 gm sodium tungstate ($Na_2WO_4 \cdot 2H_2O$—Mallinckrodt, Folin Special) in distilled water and dilute to 100 ml. The solution must be neutral or slightly alkaline to phenolphthalein, not more than 0.4 ml being required to neutralize 10 ml 0.1 N hydrochloric acid. Preserve in resistant-glass bottle.
2. **Sulfuric Acid, Volumetric 2/3 N.** Slowly add 20 ml concentrated sulfuric acid (H_2SO_4—Grasselli, A.C.S.) to 1000 ml distilled water and mix well; titrate against standard 1 N alkali using phenolphthalein as indicator; adjust accordingly and re-titrate—20 ml of 2/3 N acid require 13.3 ml of 1 N alkali.

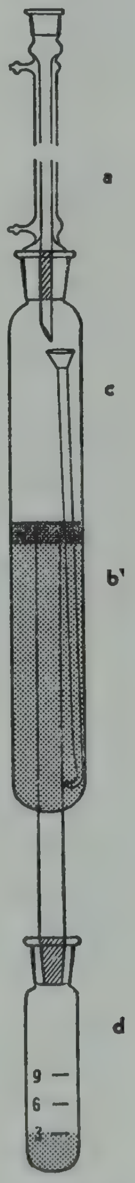

Fig. P-10.

3. **Sulfuric Acid, Volumetric 10 N.** Slowly add 300 ml concentrated sulfuric acid (H_2SO_4—Grasselli, A.C.S.) to 750 ml distilled water; mix well and allow to cool; dilute 10 ml of this approximately 10 N solution to 100 ml; titrate against standard 1 N alkali using phenolphthalein as indicator; adjust accordingly and re-titrate—20 ml of a 1:10 dilution of 10 N acid require 20 ml of 1 N alkali. Preserve in Pyrex glass-stoppered bottle.
4. **Ether, Anhydrous** ($(C_2H_5)_2O$—Mallinckrodt, A.C.S.). For maximum accuracy, the ether should be re-distilled daily from an all-glass Pyrex distillation apparatus as chromogenic substances rapidly develop on exposure. Preserve in a brown Pyrex glass-stoppered bottle.
5. **Ethyl Alcohol, 95%** (C_2H_5OH—U. S. Industrial Chemicals, U.S.P.).
6. **Phenol Standard (2 ml contain 0.005 mg).** In a 100 ml volumetric flask place 12.5 ml of the dilute standard (Reagent #10b) and dilute to volume with distilled water. Prepare fresh daily.
7. **p-Nitroaniline Reagent (Theis-Benedict).** Just prior to use add 1.5 ml 5% sodium nitrite (a) to 25 ml p-nitroaniline solution (b) and mix thoroughly. The solution must be perfectly clear and cannot be used when a turbidity develops.
   (a) Sodium nitrite, 5% aqueous; dissolve 5 gm granular sodium nitrite ($NaNO_2$—Mallinckrodt, AR) in distilled water and dilute to 100 ml. Preserve in refrigerator.
   (b) p-Nitroaniline solution; in a 500 ml mixing cylinder place 400 ml distilled water; add 40 ml concentrated hydrochloric acid (HCl—Baker & Adamson, A.C.S.); mix by inversion; adjust to volume with distilled

water; add 1.5 gm p-nitroaniline ($NO_2C_6H_4NH_2$—Eastman #179) and shake until dissolved.

8. **Sodium Carbonate, 5% Aqueous.** Add 50 gm anhydrous sodium carbonate (Na_2CO_3—Baker & Adamson, A.C.S.) to approximately 900 ml distilled water; shake until dissolved and dilute to 1000 ml; allow to stand until clear; do not filter. Preserve in ceresin-lined bottle.

Technic

Note: If conjugated phenol (Method P-11a) is to be determined on the same sample, reserve the residue in the extractor in Step 13.

For concentrations of free phenol in excess of 0.125 mg per 100 ml, use 6 or 9 ml ether (4) in a large receiver in Step 10, dilute with 10 or 15 ml ethyl alcohol (5) respectively in Step 14 and take an 8 ml aliquot for analysis; correct the values obtained from the calibration table accordingly.

1. In a 100 ml centrifuge tube place
   24 ml distilled water
   12 ml whole blood and mix by stirring with a glass rod.
2. When laking is complete add 12 ml 10% sodium tungstate (1) and mix with the rod.
3. Slowly add 12 ml 2/3 N sulfuric acid (2) and mix thoroughly with the rod.
4. Allow to stand for 15 minutes, stirring occasionally—the mixture should be foam-free.
5. Centrifuge at 3000 rpm for 15 minutes—if the centrifugate is not water-clear, decant the supernatant fluid into small centrifuge tubes and recentrifuge—FILTRATION IS NOT PERMISSIBLE.
6. Transfer 40 ml clear centrifugate to a coaxial extraction tube.
7. Add 1 ml 10 N sulfuric acid (3).
8. Add freshly distilled ether (4) until the ether layer just overflows the inner return tube—about 6–9 ml are required.
9. Attach the extraction tube to the condenser and place a solid glass stopper loosely in the top of the condenser.
10. In the small graduated receiver place a few particles of pulverized porcelain, 3 ml freshly distilled ether (4) and attach to the extraction tube. Note: The large receiver is used only with 6 or 9 ml ether for excessive concentrations of phenol.
11. Immerse the receiver in a water bath maintained at 70–75° C. and allow extraction to proceed for 2 hours. Note: Do not allow any of the fluid to spill down the inner return tube.
12. Remove from water bath and gently shake the apparatus until sufficient ether overflows the inner return tube to bring the volume in the receiver to slightly less than 3 ml.
13. Detach receiver and adjust the volume to exactly 3 ml with freshly distilled ether (4)—RESERVE RESIDUE IN EXTRACTOR FOR CONJUGATED PHENOL ANALYSIS (Method P-11a).
14. To the extract in the receiver add 5 ml 95% ethyl alcohol (5).
15. Decant the alcohol-ether solution into a cuvette (sample) and add 2 ml phenol standard (6).
16. In another cuvette (reagent blank) place
    3 ml freshly distilled ether (4)
    5 ml 95% ethyl alcohol (5)
    2 ml distilled water and mix.
17. To each cuvette add 1 ml freshly prepared p-nitroaniline reagent (7) and mix.
18. Allow to stand at room temperature for 1 minute.
19. Add 3 ml 5% sodium carbonate (8) and mix.
20. Measure the transmittancy of the sample against the reagent blank set at 100% transmission at a wave length of **500 mμ** and obtain the apparent concentration of free phenol from the calibration table.
21. Calculate the true concentration of free phenol as follows.

Calculation

$$\frac{\text{Apparent concentration from calibration table}}{0.76} = \text{Free phenol in mg per 100 ml blood}$$

CALIBRATION PROCEDURE

Special Apparatus

Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.

Reagents

4. **Ether, Anhydrous.** Same as for Test Procedure.
5. **Ethyl Alcohol, 95%.** Same as for Test Procedure.
6. **Phenol Standard (2 ml contain 0.005 mg).** Same as for Test Procedure.
7. **p-Nitroaniline Reagent (Theis-Benedict).** Same as for Test Procedure.
8. **Sodium Carbonate, 5% Aqueous.** Same as for Test Procedure.
9. **Hydrochloric Acid, Volumetric 0.1 N.** Add 10 ml concentrated hydrochloric acid (HCl—Baker & Adamson, A.C.S.) to 1000 ml distilled water and mix; standardize against 0.1 N alkali using phenolphthalein as indicator.
10. **Phenol Standards**
    (a) Phenol stock standard (1 ml contains 1 mg phenol). In a 1000 ml volumetric flask place slightly more than 1 gm crystalline phenol (C_6H_5OH—Baker & Adamson, Acid Carbolic, Reagent, Loose Crystals); add about 800 ml 0.1 N hydrochloric acid (9), shake until dissolved and dilute to volume with same. In a 250 ml glass-stoppered Erlenmeyer flask place 25 ml of the phenol solution; add 50 ml 0.1 N sodium hy-

droxide and heat to 65° C.; add 25 ml 0.1 N iodine and mix; stopper the flask and allow to stand at room temperature for 45 minutes; add 5 ml concentrated hydrochloric acid and titrate the excess iodine with 0.1 N sodium thiosulfate using starch as indicator—1 ml of 0.1 N iodine is equivalent to 1.567 mg phenol; adjust accordingly and re-titrate—the excess iodine after treatment of 25 ml phenol standard as outlined should require 9.05 ml sodium thiosulfate (i.e., 15.95 ml iodine combine with 25 mg phenol). Preserve in refrigerator. Stability indefinite.

(b) Dilute standard (1 ml contains 0.02 mg phenol). In a 100 ml volumetric flask place 2 ml stock standard (10a) and dilute to volume with distilled water. Prepare just before use.

(c) Calibration standards. In a series of six accurately calibrated 50 ml volumetric flasks place respectively 0, 1, 2, 3, 4 and 5 ml of the dilute standard (10b) and dilute to volume with 95% ethyl alcohol (5). These standards represent concentrations of phenol equivalent to 0, 0.025, 0.050, 0.075, 0.100 and 0.125 mg per 100 ml sample. Prepare just before use and proceed immediately as follows.

Technic

1. Transfer 5 ml of each calibration standard (10c) to a correspondingly marked cuvette.
2. To each cuvette add 3 ml freshly distilled ether (4) and mix.
3. Add 2 ml *phenol standard* (*6*) and mix.
4. Proceed exactly as outlined in the Test Procedure, Steps 16–19 inclusive.
5. Set the reagent blank at 100% transmission at a wave length of **500** mμ and record the transmittancy of the 0, 0.025, 0.050, 0.075, 0.100 and 0.125 mg per cent standards. The transmittancy of the zero concentration standard should be approximately 70% and that of the 0.125 mg standard approximately 37%.
6. Plot the observed values on semi-log graph paper—the points fall on a straight line which passes through the transmission axis near 70% transmission (Calibration Curve P-10). Note: Because of the addition of a known quantity of phenol to each calibration standard, the calibration curve does not pass through the origin; since the same amount of phenol is added to the unknown, correction for this increment is unnecessary.
7. Prepare a calibration table from the T-C values obtained from the plotted curve.

Method P-10b

DETERMINATION IN URINE

SAMPLE

Aliquot of a 24-hour specimen. Keep cool during collection. The determination cannot be carried out during the administration of sulfonamides.

TEST PROCEDURE

Special Apparatus

As listed under the Test Procedure for blood.

Reagents

4. **Ether, Anhydrous.** Same as for blood.
5. **Ethyl Alcohol, 95%.** Same as for blood.
6. **Phenol Standard (2 ml contain 0.005 mg).** Same as for blood.
7. **p-Nitroaniline Reagent (Theis-Benedict).** Same as for blood.
8. **Sodium Carbonate, 5% Aqueous.** Same as for blood.
11. **Sodium Hydroxide, Volumetric 0.1 N.** Prepare by diluting slightly more than the required amount of concentrated carbonate-free sodium hydroxide solution (see Appendix) and standardize against 0.1 N acid using phenolphthalein as indicator. Preserve in ceresin-lined bottle.
12. **Wide Range Indicator Paper** (Hydrion).

Technic

Note: If conjugated phenol (Method P-11b) is to be determined on the same sample, reserve the residue in the extractor in Step 10.

For concentrations of free phenol in excess of 0.125 mg per 100 ml, use 6 or 9 ml ether (4) in a large receiver in Step 7, dilute with 10 or 15 ml ethyl alcohol (5) respectively in Step 11 and take an 8 ml aliquot for analysis; correct the values obtained from the calibration table for blood accordingly.

1. In a 100 ml volumetric flask place
   20 ml filtered urine
   30 ml distilled water and mix.
2. Adjust to pH 10.0 (±0.2) with 0.1 N sodium hydroxide (11) using wide range indicator paper (12).
3. Dilute to volume with distilled water and mix.
4. Transfer 40 ml diluted urine to a coaxial extraction tube.
5. Add freshly distilled ether (4) until the ether layer just overflows the inner return tube—about 6–9 ml are required.
6. Attach the extraction tube to the condenser and place a solid glass stopper loosely in the top of the condenser.
7. In the small graduated receiver place a few particles of pulverized porcelain, 3 ml freshly distilled ether (4) and attach to the extraction tube.

Note: The large receiver is used only with 6 or 9 ml ether for excessive concentrations of phenol.

8. Immerse the receiver in a water bath maintained at 70–75° C. and allow extraction to proceed for 2 hours. Note: Do not allow any of the fluid to spill down the inner return tube.
9. Remove from water bath and gently shake the apparatus until sufficient ether overflows the inner return tube to bring the volume in the receiver to slightly less than 3 ml.
10. Detach receiver and adjust the volume to exactly 3 ml with freshly distilled ether (4)—RESERVE RESIDUE IN EXTRACTOR FOR CONJUGATED PHENOL ANALYSIS (Method P-11b).
11. To the extract in the receiver add 5 ml 95% ethyl alcohol (5).
12. Proceed exactly as outlined in the Test Procedure for blood, Steps 15–19 inclusive.
13. Within 10 minutes measure the transmittancy of the sample against the reagent blank set at 100% transmission at a wave length of **500 mμ** and obtain the concentration of free phenol in mg per 100 ml urine directly from the calibration table for blood.

Calculation

$$\frac{\text{Volume of 24-hour specimen}}{100} \times \text{Free phenol in mg per 100 ml} = \text{Mg free phenol excreted in 24 hours}$$

◆

PROCEDURE FOR USE WITH LINEAR-SCALE PHOTOELECTRIC COLORIMETERS (SHEARD-SANFORD TYPE)

TEST PROCEDURE

Exactly as outlined; set the reagent blank at 100 and use a green filter.

CALIBRATION PROCEDURE

Exactly as outlined—use a green filter; plot the observed readings on semi-log graph paper—the points fall on a straight line passing through the transmission axis.

◆

PROCEDURE FOR USE WITH LOGARITHMIC-SCALE PHOTOELECTRIC COLORIMETERS (KLETT-SUMMERSON TYPE)

TEST PROCEDURE

Exactly as outlined; set the reagent blank at 0 and use a green filter.

CALIBRATION PROCEDURE

Exactly as outlined—use a green filter; plot the observed readings on linear graph paper—the points fall on a straight line passing through the instrument reading axis.

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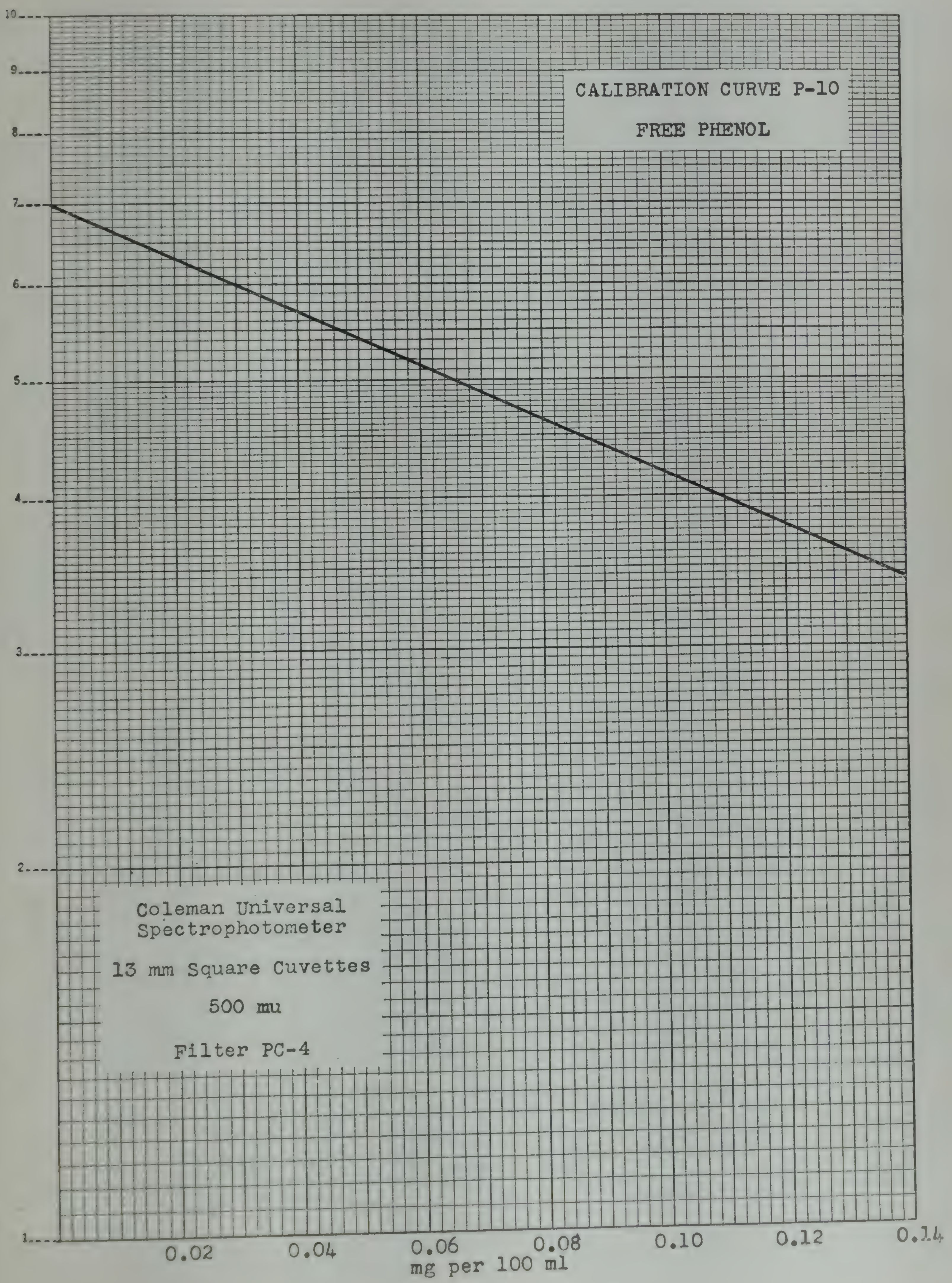
◆


CALIBRATION CURVE P-10
FREE PHENOL
Coleman Universal Spectrophotometer
13 mm Square Cuvettes
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Filter PC-4
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mg per 100 ml

Meter Reading	0	1	2	3	4	5	6	7	8	9
0										
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PHENOL, CONJUGATED

A spectrophotometric adaptation based on the methods and modifications of Moir (1922), Theis and Benedict (1924), Schmidt, Schmulovitz, Szczpinski and Wylie (1937) and Schmidt (1942; 1943)

PRINCIPLE

After removal of free phenol, the residue is hydrolyzed to liberate the conjugated phenol which is then extracted with ether and determined as outlined in the method for free phenol (Method P-10), the same analytical principles being involved.

DETERMINATION IN WHOLE BLOOD

SAMPLE

Blood withdrawn during post-absorptive state. Anticoagulant—potassium oxalate. The determination cannot be carried out during the administration of sulfonamides.

TEST PROCEDURE

Special Apparatus

Centrifuge tubes, 100 ml.

Schmidt-Wylie continuous extraction assembly (Fig. P-10). For a more detailed description see Method P-10.

Boiling flask, 100 ml, round bottom with glass joint and water-cooled condenser.

Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.

Reagents

1. **Sodium Tungstate, 10% Aqueous.** Same as for free phenol.
2. **Sulfuric Acid, Volumetric 2/3 N.** Same as for free phenol.
3. **Sulfuric Acid, Volumetric 10 N.** Same as for free phenol.
4. **Ether, Anhydrous.** Same as for free phenol.
5. **Ethyl Alcohol, 95%.** Same as for free phenol.
6. **Phenol Standard (2 ml contain 0.005 mg).** Same as for free phenol.
7. **p-Nitroaniline Reagent (Theis-Benedict).** Same as for free phenol.
8. **Sodium Carbonate, 5% Aqueous.** Same as for free phenol.

Technic

Note: If conjugated phenol is to be determined on the residue as reserved in the determination of free phenol (Method P-10a, Step 13), omit the following Steps 1–12 inclusive.

For concentrations of conjugated phenol in excess of 0.125 mg per 100 ml, use 6 or 9 ml ether (4) in a large receiver in Step 21, dilute with 10 or 15 ml ethyl alcohol (5) respectively in Step 25 and take an 8 ml aliquot for analysis; correct the values obtained from the calibration table for free phenol (Method P-10) accordingly.

1. In a 100 ml centrifuge tube place
   24 ml distilled water
   12 ml whole blood and mix by stirring with a glass rod.
2. When laking is complete add 12 ml 10% sodium tungstate (1) and mix with the rod.
3. Slowly add 12 ml 2/3 N sulfuric acid (2) and mix thoroughly with the rod.
4. Allow to stand for 15 minutes, stirring occasionally—the mixture should be foam-free.
5. Centrifuge at 3000 rpm for 15 minutes—if the centrifugate is not water-clear, decant the supernatant fluid into small centrifuge tubes and recentrifuge—FILTRATION IS NOT PERMISSIBLE.
6. Transfer 40 ml clear centrifugate to a coaxial extraction tube.
7. Add 1 ml 10 N sulfuric acid (3).
8. Add freshly distilled ether (4) until the ether layer just overflows the inner return tube—about 6–9 ml are required.
9. Attach the extraction tube to the condenser and place a solid glass stopper loosely in the top of the condenser.
10. In the small graduated receiver place a few particles of pulverized porcelain, 3 ml freshly distilled ether (4) and attach to the extraction tube.
11. Immerse the receiver in a water bath maintained at 70–75° C. and allow extraction to proceed for 2 hours. Note: Do not allow any of the fluid to spill down the inner return tube.
12. Remove from water bath and gently shake the apparatus until sufficient ether overflows the inner return tube to bring the volume in the receiver to slightly less than 3 ml.
13. Detach receiver—RESERVE EXTRACT IN RECEIVER FOR FREE PHENOL ANALYSIS (Method P-10a).
14. With a capillary pipette draw off most of the ether layer from the extractor and discard. Note: The ether should be removed from the extractor immediately after completing the extraction of free phenol since prolonged contact with the sample may produce chromogenic substances.
15. Decant the extracted residue in the extractor into a 100 round-bottom boiling flask with ground joint.
16. Add a few glass beads and immerse in a water bath at 70–75° C. until the ether has completely volatilized.
17. Immediately connect the condenser and reflux gently for 1 hour.

18. Allow to cool and pour the hydrolysate back into the original extractor.
19. Add freshly distilled ether (4) until the ether layer just overflows the inner return tube—about 6–9 ml are required.
20. Attach the extraction tube to the condenser and place a solid glass stopper loosely in the top of the condenser.
21. In the small graduated receiver place a few particles of pulverized porcelain, 3 ml freshly distilled ether (4) and attach to the extraction tube. Note: The large receiver is used only with 6 or 9 ml ether for excessive concentrations of phenol.
22. Immerse the receiver in a water bath maintained at 70–75° C. and allow extraction to proceed for 2 hours. Note: Do not allow any of the fluid to spill down the inner return tube.
23. Remove from water bath and gently shake the apparatus until sufficient ether overflows the inner return tube to bring the volume in the receiver to slightly less than 3 ml.
24. Detach receiver and adjust the volume to exactly 3 ml with freshly distilled ether (4).
25. To the extract in the receiver add 5 ml 95% ethyl alcohol (5).
26. Decant the alcohol-ether solution into a cuvette (sample) and add 2 ml phenol standard (6).
27. In another cuvette (reagent blank) place
    3 ml freshly distilled ether (4)
    5 ml 95% ethyl alcohol (5)
    2 ml distilled water and mix.
28. To each cuvette add 1 ml freshly prepared p-nitroaniline reagent (7) and mix.
29. Allow to stand at room temperature for 1 minute.
30. Add 3 ml 5% sodium carbonate (8) and mix.
31. Measure the transmittancy of the sample against the reagent blank set at 100% transmission at a wave length of 500 mμ and obtain the apparent concentration of conjugated phenol from the calibration table for free phenol (Method P-10).
32. Calculate the true concentration of conjugated phenol as follows:

Calculation

$$\frac{\text{Apparent concentration from calibration table}}{0.76} = \text{Conjugated phenol in mg per 100 ml blood}$$

CALIBRATION PROCEDURE

Values are obtained directly from the calibration table for free phenol (Method P-10).

Method P-11b

DETERMINATION IN URINE

SAMPLE

Aliquot of a 24-hour specimen. Keep cool during collection. The determination cannot be carried out during the administration of sulfonamides.

TEST PROCEDURE

Special Apparatus

As listed under the Test Procedure for blood.

Reagents

3. **Sulfuric Acid, Volumetric 10 N.** Same as for blood.
4. **Ether, Anhydrous.** Same as for blood.
5. **Ethyl Alcohol, 95%.** Same as for blood.
6. **Phenol Standard (2 ml contain 0.005 mg).** Same as for blood.
7. **p-Nitroaniline Reagent (Theis-Benedict).** Same as for blood.
8. **Sodium Carbonate, 5% Aqueous.** Same as for blood.
11. **Sodium Hydroxide, Volumetric 0.1 N.** Same as for free phenol in urine.
12. **Wide Range Indicator Paper.** Same as for free phenol in urine.
13. **Sodium Hydroxide, Volumetric 1 N.** Prepare by diluting slightly more than the required amount of concentrated carbonate-free sodium hydroxide solution (see Appendix) and standardize against 1 N acid using phenolphthalein as indicator. Preserve in ceresin-lined bottle.

Technic

Note: If conjugated phenol is to be determined on the residue as reserved in the determination of free phenol (Method P-10b, Step 10), omit the following Steps 1–10 inclusive.

For concentrations of conjugated phenol in excess of 5 mg per 100 ml, use 6 or 9 ml ether (4) in a large receiver in Step 23, dilute with 10 or 15 ml ethyl alcohol (5) respectively in Step 27 and take an 8 ml aliquot for analysis; correct the values obtained from the calibration table for free phenol in blood (Method P-10) accordingly.

1. In a 100 ml volumetric flask place
    1 ml filtered urine
    50 ml distilled water and mix.
2. Adjust to pH 10.0 (±0.2) with 0.1 N sodium hydroxide (11) using wide range indicator paper (12).
3. Dilute to volume with distilled water and mix.
4. Transfer 40 ml diluted urine to a coaxial extraction tube.
5. Add freshly distilled ether (4) until the ether layer just overflows the inner return tube—about 6–9 ml are required.

6. Attach the extraction tube to the condenser and place a solid glass stopper loosely in the top of the condenser.
7. In the small graduated receiver place a few particles of pulverized porcelain, 3 ml freshly distilled ether (4) and attach to the extraction tube.
8. Immerse the receiver in a water bath maintained at 70–75° C. and allow extraction to proceed for 2 hours. Note: Do not allow any of the fluid to spill down the inner return tube.
9. Remove from water bath and gently shake the apparatus until sufficient ether overflows the inner return tube to bring the volume in the receiver to slightly less than 3 ml.
10. Detach receiver—RESERVE EXTRACT IN RECEIVER FOR FREE PHENOL ANALYSIS (Method P-10b).
11. With a capillary pipette draw off most of the ether layer from the extractor and discard. Note: The ether should be removed from the extractor immediately after completing the extraction of free phenol since prolonged contact with the sample may produce chromogenic substances.
12. Decant the extracted residue in the extractor into a 100 ml round-bottom boiling flask with ground joint.
13. Rinse the extraction tube with two 10 ml portions distilled water, pouring the washings into the boiling flask.
14. Adjust to pH 1.0 with 10 N sulfuric acid (3) using wide range indicator paper (12)—approximately 1 ml is required.
15. Add a few glass beads and immerse in a water bath at 70–75° C. until the ether has completely volatilized.
16. Immediately connect the condenser and reflux gently for 1 hour.
17. Allow to cool and quantitatively transfer the hydrolysate to an accurately calibrated 100 ml mixing cylinder.
18. Adjust to pH 10.0 (±0.2) with 1 N sodium hydroxide (13) using wide range indicator paper (12) —approximately 11.2 ml are required.
19. Dilute to 80 ml with distilled water and mix.
20. Transfer 40 ml diluted hydrolysate back into the original extractor.
21. Proceed exactly as outlined in the Test Procedure for blood, Steps 19–30 inclusive.
22. Measure the transmittancy of the sample against the reagent blank set at 100% transmission at a wave length of **500** mμ and multiply the value from the calibration table for free phenol in blood (Method P-10) by 40 to obtain the concentration of conjugated phenol in mg per 100 ml urine.

Calculation

$$\frac{\text{Volume of 24-hour specimen}}{100} \times \text{Conjugated phenol in mg per 100 ml} = \text{Mg conjugated phenol excreted in 24 hours}$$

PROCEDURE FOR USE WITH LINEAR-SCALE PHOTOELECTRIC COLORIMETERS (SHEARD-SANFORD TYPE)

TEST PROCEDURE

Exactly as outlined; set the reagent blank at 100 and use a green filter.

CALIBRATION PROCEDURE

Need not be performed; values obtained from the calibration table for free phenol.

PROCEDURE FOR USE WITH LOGARITHMIC-SCALE PHOTOELECTRIC COLORIMETERS (KLETT-SUMMERSON TYPE)

TEST PROCEDURE

Exactly as outlined; set the reagent blank at 0 and use a green filter.

CALIBRATION PROCEDURE

Need not be performed; values obtained from the calibration table for free phenol.

BIBLIOGRAPHY

Same as listed under Free Phenol (Method P-10).

NOTES

PHENOL, TOTAL

A spectrophotometric adaptation based on the methods and modifications of Moir (1922), Theis and Benedict (1924), Schmidt, Schmulovitz, Szczpinski and Wylie (1937) and Schmidt (1942; 1943)

Either of two methods may be used for the estimation of total phenol—by difference or by actual determination.

A. By Difference

1. Determine the concentration of free phenol as outlined in Method P-10.
2. Determine the concentration of conjugated phenol as outlined in Method P-11.
3. Calculation—
Free phenol in mg per 100 ml + Conjugated phenol in mg per 100 ml = Total phenol in mg per 100 ml

B. By Determination

PRINCIPLE

After a preliminary hydrolysis to liberate conjugated phenol, the original free phenol and the liberated phenol (constituting total phenol) are determined as outlined in the method for free phenol (Method P-10), the same analytical principles being involved.

DETERMINATION IN WHOLE BLOOD

SAMPLE

Blood withdrawn during post-absorptive state. Anticoagulant—potassium oxalate. The determination cannot be carried out during the administration of sulfonamides.

TEST PROCEDURE

Special Apparatus

Centrifuge tubes, 100 ml.

Boiling flask, 100 ml, round bottom with glass joint and water-cooled condenser.

Schmidt-Wylie continuous extraction assembly (Fig. P-10). For a more detailed description see Method P-10.

Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.

Reagents

1. **Sodium Tungstate, 10% Aqueous.** Same as for free phenol.
2. **Sulfuric Acid, Volumetric 2/3 N.** Same as for free phenol.
3. **Sulfuric Acid, Volumetric 10 N.** Same as for free phenol.
4. **Ether, Anhydrous.** Same as for free phenol.
5. **Ethyl Alcohol, 95%.** Same as for free phenol.
6. **Phenol Standard (2 ml contain 0.005 mg).** Same as for free phenol.
7. **p-Nitroaniline Reagent (Theis-Benedict).** Same as for free phenol.
8. **Sodium Carbonate, 5% Aqueous.** Same as for free phenol.

Technic

Note: For concentrations of total phenol in excess of 0.25 mg per 100 ml, use 6 or 9 ml ether (4) in a large receiver in Step 12, dilute with 10 or 15 ml ethyl alcohol (5) respectively in Step 16 and take an 8 ml aliquot for analysis; correct the values obtained from the calibration table for free phenol (Method P-10) accordingly.

1. In a 100 ml centrifuge tube place
   24 ml distilled water
   12 ml whole blood and mix by stirring with a glass rod.
2. When laking is complete add 12 ml 10% sodium tungstate (1) and mix with the rod.
3. Slowly add 12 ml 2/3 N sulfuric acid (2) and mix thoroughly with the rod.
4. Allow to stand for 15 minutes, stirring occasionally—the mixture should be foam-free.
5. Centrifuge at 3000 rpm for 15 minutes—if the centrifugate is not water-clear, decant the supernatant fluid into small centrifuge tubes and recentrifuge—FILTRATION IS NOT PERMISSIBLE.
6. Transfer 40 ml clear centrifugate to a 100 ml round-bottom boiling flask with ground joint.
7. Add a few glass beads and 1 ml 10 N sulfuric acid (3).
8. Connect the condenser and reflux gently for 1 hour.
9. Allow to cool and pour the hydrolysate into a coaxial extraction tube.
10. Add freshly distilled ether (4) until the ether layer just overflows the inner return tube—about 6–9 ml are required.
11. Attach the extraction tube to the condenser and place a solid glass stopper loosely in the top of the condenser.
12. In the small graduated receiver place a few particles of pulverized porcelain, 3 ml freshly distilled ether (4) and attach to the extraction tube. Note: The large receiver is used only with 6 or 9 ml ether for excessive concentrations of phenol.
13. Immerse the receiver in a water bath maintained at 70–75° C. and allow extraction to proceed for

2 hours. Note: Do not allow any of the fluid to spill down the inner return tube.

14. Remove from water bath and gently shake the apparatus until sufficient ether overflows the inner return tube to bring the volume in the receiver to slightly less than 3 ml.
15. Detach receiver and adjust the volume to exactly 3 ml with freshly distilled ether (4).
16. In a cuvette (sample) place
    1.5 ml ether extract
    1.5 ml freshly distilled ether (4)
    5 ml 95% ethyl alcohol (5)
    2 ml phenol standard (6) and mix.
17. In another cuvette (reagent blank) place
    3 ml freshly distilled ether (4)
    5 ml 95% ethyl alcohol (5)
    2 ml distilled water and mix.
18. To each cuvette add 1 ml freshly prepared p-nitroaniline reagent (7) and mix.
19. Allow to stand at room temperature for 1 minute.
20. Add 3 ml 5% sodium carbonate (8) and mix.
21. Measure the transmittancy of the sample against the reagent blank set at 100% transmission at a wave length of 500 mμ and multiply the value from the calibration table for free phenol (Method P-10) by 2 to obtain the apparent concentration of total phenol.
22. Calculate the true concentration of total phenol as follows:

Calculation

$$\frac{\text{Apparent concentration from calibration table}}{0.76} = \text{Total phenol in mg per 100 ml blood}$$

CALIBRATION PROCEDURE

The values in the calibration table for free phenol (Method P-10), when multiplied by 2 are equivalent to the apparent concentration of total phenol in mg per 100 ml sample.

Method P-12b

DETERMINATION IN URINE

SAMPLE

Aliquot of a 24-hour specimen. Keep cool during collection. The determination cannot be carried out during the administration of sulfonamides.

TEST PROCEDURE

Special Apparatus

Centrifuge tubes, 100 ml.
Boiling flask, 200 ml, round bottom with glass joint and water-cooled condenser.
Schmidt-Wylie continuous extraction assembly (Fig. P-10). For a more detailed description see Method P-10.
Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.

Reagents

3. **Sulfuric Acid, Volumetric 10 N.** Same as for blood.
4. **Ether, Anhydrous.** Same as for blood.
5. **Ethyl Alcohol, 95%.** Same as for blood.
6. **Phenol Standard (2 ml contain 0.005 mg).** Same as for blood.
7. **p-Nitroaniline Reagent (Theis-Benedict).** Same as for blood.
8. **Sodium Carbonate, 5% Aqueous.** Same as for blood.
12. **Wide Range Indicator Paper.** Same as for conjugated phenol in urine.
13. **Sodium Hydroxide, Volumetric 1 N.** Same as for conjugated phenol in urine.

Technic

Note: For concentrations of total phenol in excess of 5 mg per 100 ml, use less hydrolysate in Step 5 and correct the values obtained from the calibration table for free phenol in blood (Method P-10) accordingly.

1. In a 100 ml volumetric flask place
    1 ml filtered urine
    1 ml 10 N sulfuric acid (3).
2. Dilute to volume with distilled water and mix.
3. Quantitatively transfer the contents of the flask to a 200 ml round-bottom boiling flask with ground joint.
4. Add a few glass beads, connect condenser and reflux gently for 1 hour.
5. Allow to cool and transfer 50 ml hydrolysate to a 100 ml volumetric flask.
6. Add 35 ml distilled water and adjust to pH 10.0 (±0.2) by the addition of 1 N sodium hydroxide (13) using wide range indicator paper (12)—approximately 11.7 ml are required.
7. Dilute to volume with distilled water and mix.
8. Transfer 40 ml of the alkalinized hydrolysate to a coaxial extraction tube.
9. Add freshly distilled ether (4) until the ether layer just overflows the inner return tube—about 6–9 ml are required.
10. Attach the extraction tube to the condenser and place a solid glass stopper loosely in the top of the condenser.
11. In the small graduated receiver place a few parti-

cles of pulverized porcelain, 3 ml freshly distilled ether (4) and attach to the extraction tube.

12. Immerse the receiver in a water bath maintained at 70–75° C. and allow extraction to proceed for 2 hours. Note: Do not allow any of the fluid to spill down the inner return tube.
13. Remove from water bath and gently shake the apparatus until sufficient ether overflows the inner return tube to bring the volume in the receiver to slightly less than 3 ml.
14. Detach receiver and adjust the volume to exactly 3 ml with freshly distilled ether (4).
15. To the extract in the receiver add 5 ml 95% ethyl alcohol (5).
16. Decant the alcohol-ether solution into a cuvette (sample) and add 2 ml phenol standard (6).
17. Proceed exactly as outlined in the Test Procedure for blood, Steps 17–20 inclusive.
18. Measure the transmittancy of the sample against the reagent blank set at 100% transmission at a wave length of **500** mμ and multiply the value from the calibration table for free phenol in blood (Method P-10) by 40 to obtain the concentration of total phenol in mg per 100 ml urine.

Calculation

$$\frac{\text{Volume of 24-hour specimen}}{100} \times \text{Total phenol in mg per 100 ml} = \text{Mg total phenol excreted in 24 hours}$$

PROCEDURE FOR USE WITH LINEAR-SCALE PHOTOELECTRIC COLORIMETERS (SHEARD-SANFORD TYPE)

TEST PROCEDURE

Exactly as outlined; set the reagent blank at 100 and use a green filter.

CALIBRATION PROCEDURE

Need not be performed; values obtained from the calibration table for free phenol.

PROCEDURE FOR USE WITH LOGARITHMIC-SCALE PHOTOELECTRIC COLORIMETERS (KLETT-SUMMERSON TYPE)

TEST PROCEDURE

Exactly as outlined; set the reagent blank at 0 and use a green filter.

CALIBRATION PROCEDURE

Need not be performed; values obtain from the calibration table for free phenol.

BIBLIOGRAPHY

Same as listed under Free Phenol (Method P-10).

NOTES

NOTES

PHENOLSULFONPHTHALEIN

A spectrophotometric adaptation based on the methods and modifications of Rowntree and Geraghty (1912) and Burwell and Jones (1921)

PRINCIPLE

Phenolsulfonphthalein, upon alkalinization with sodium hydroxide, is converted to the red monosodium salt and determined as such. When necessary, interfering blood and/or biliary pigments are removed by treatment with zinc acetate.

DETERMINATION IN URINE

SAMPLE

Two urine specimens collected in separate containers exactly one hour and two hours after intravenous injection or one hour and ten minutes and two hours and ten minutes after intramuscular injection of 6 mg phenolsulfonphthalein. Make certain that the bladder is completely emptied after each collection. The volume of each specimen must be 40 ml or more for reliable results.

TEST PROCEDURE

Special Apparatus

Mixing cylinders—1000 ml, glass-stoppered.
Capillary droppers delivering 40 drops per ml.
Graduated test tubes—10 ml in 0.1 ml; the 10 ml graduation must be accurately calibrated. (Required only for Procedure B).
Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.

Reagents

1. **Phosphoric Acid,** 85% (H_3PO_4—Baker & Adamson, A.C.S.).
2. **Sodium Hydroxide, 10% Aqueous.** Dissolve 10 gm sodium hydroxide pellets (NaOH—Baker & Adamson, A.C.S.) in distilled water and dilute to 100 ml. Preserve in ceresin-lined bottle.
3. **Zinc Acetate, Saturated Alcoholic.** In a four-ounce brown glass bottle place 5 gm crystalline zinc acetate ($Zn(C_2H_3O_2)_2 \cdot 2H_2O$—Baker & Adamson, Reagent); add 100 ml absolute methyl alcohol (CH_3OH—Merck, A.C.S.) and shake at intervals; allow to settle and use supernatant fluid. (Required only for Procedure B).

Technic

A. For Urine Containing No Blood and/or Bile Pigments

1. Filter each hourly urine specimen in its entirety into separate, correspondingly marked 1000 ml mixing cylinders.
2. Dilute to 500 ml with distilled water and mix by inversion.
3. Transfer, by means of volumetric pipettes, exactly 5 ml from each cylinder to correspondingly marked cuvettes (sample blanks) and add 5 ml distilled water.
4. Test the reaction of the diluted urine in the cuvettes with litmus paper—if alkaline, add 1 small drop phosphoric acid (1) and mix.
5. To the remainder of diluted urine in each cylinder add 10% sodium hydroxide (2) until the maximum color is developed (do not use an excess—10 ml are usually sufficient but occasionally more may be required).
6. Dilute to 1000 ml with distilled water and mix by inversion.
7. Transfer 10 ml of the alkalinized urines to correspondingly marked cuvettes (samples).
8. Measure the transmittancy of each sample against the corresponding sample blank set at 100% transmission at a wave length of **545** $m\mu$ and obtain the concentration of phenolsulfonphthalein from the calibration table.

Note: Since 10 ml of diluted urine are removed for use as sample blanks, the sum of the determined concentrations represents only 98% of the true dye excretion. However, for clinical purposes a correction of 2% is insignificant.

Method P-20b

B. For Urine Containing Blood and/or Bile Pigments

1. Filter each hourly urine specimen in its entirety into separate, correspondingly marked 1000 ml mixing cylinders.
2. Dilute to 500 ml with distilled water and mix by inversion.
3. Transfer 20 ml diluted urine from each cylinder to correspondingly marked 125 ml Erlenmeyer flasks.
4. Add 20 ml saturated alcoholic zinc acetate (3).
5. Shake thoroughly and allow to stand for 10 minutes.
6. Filter through dry papers into correspondingly marked dry test tubes.

7. Transfer 5 ml from each tube to accurately graduated 10 ml test tubes (sample blanks).
8. Transfer a second 5 ml portion to another set of 10 ml test tubes (samples).
9. To the sample tubes add 10% sodium hydroxide (2), drop by drop, until a maximum color is attained.
10. Dilute the contents of all tubes to exactly 10 ml with distilled water and mix.
11. Allow to stand for 5 minutes.
12. Transfer the contents of each tube (filtering those necessary) to correspondingly marked cuvettes.
13. Measure the transmittancy of each sample against the corresponding sample blank set at 100% transmission at a wave length of **545** mμ and multiply the value from the calibration table by 2 to obtain the concentration of phenolsulfonphthalein in per cent.

Calculation

(Concentration of phenolsulfonphthalein in sample I in % + Concentration of phenolsulfonphthalein in sample II in %) × 0.06 = Mg phenolsulfonphthalein excreted

CALIBRATION PROCEDURE

Note: The following calibration procedure is based on the premise that 6 mg of dye are administered in the renal function test.

Special Apparatus

Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.

Reagents

2. **Sodium Hydroxide, 10% Aqueous.** Same as for Test Procedure.
4. **Phenolsulfonphthalein Standards**
    (a) Phenolsulfonphthalein stock standard (1 ml contains 6 mg monosodium salt of phenolsulfonphthalein). This standard solution, which is the same as that used for injection, may be secured in ampoules (Hynson, Westcott & Dunning) containing slightly more than 1 ml, thus allowing withdrawal of an accurate 1 ml portion.
    (b) Dilute standard (1 ml contains 0.006 mg phenolsulfonphthalein). In a 1000 ml volumetric flask place 1 ml stock standard (4a); add approximately 500 ml distilled water and 2 ml 10% sodium hydroxide (2); dilute to volume with distilled water. Use shortly after preparation as the color gradually fades. It may be preserved for a limited period in a ceresin-lined bottle.
    (c) Calibration standards. In a series of eleven cuvettes place respectively 0, 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10 ml of the dilute standard (4b); add 10, 9, 8, 7, 6, 5, 4, 3, 2, 1 and 0 ml distilled water respectively, bringing the volume in each cuvette to 10 ml. These standards represent concentrations of phenolsulfonphthalein equivalent to 0, 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100% dye excretion. Prepare just before use and proceed immediately as follows.

Technic

1. Set the zero concentration standard at 100% transmission at a wave length of **545** mμ and record the transmittancy of the 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100% standards. The transmittancy of the 100% standard should be approximately 14%.
2. Plot the observed values on semi-log graph paper—the points fall on a smooth curve which passes through the origin (Calibration Curve P-20).
3. Prepare a calibration table from the T-C values obtained from the plotted curve.

PROCEDURE FOR USE WITH LINEAR-SCALE PHOTOELECTRIC COLORIMETERS (SHEARD-SANFORD TYPE)

TEST PROCEDURE

Exactly as outlined; set the sample blank at 100 and use a green filter.

CALIBRATION PROCEDURE

Exactly as outlined—use a green filter; plot the observed readings on semi-log graph paper—the points fall on a slight curve passing through the origin.

PROCEDURE FOR USE WITH LOGARITHMIC-SCALE PHOTOELECTRIC COLORIMETERS (KLETT-SUMMERSON TYPE)

TEST PROCEDURE

Exactly as outlined; set the sample blank at 0 and use a green filter.

CALIBRATION PROCEDURE

Exactly as outlined—use a green filter; plot the observed readings on linear graph paper—the points fall on a slight curve passing through the origin.

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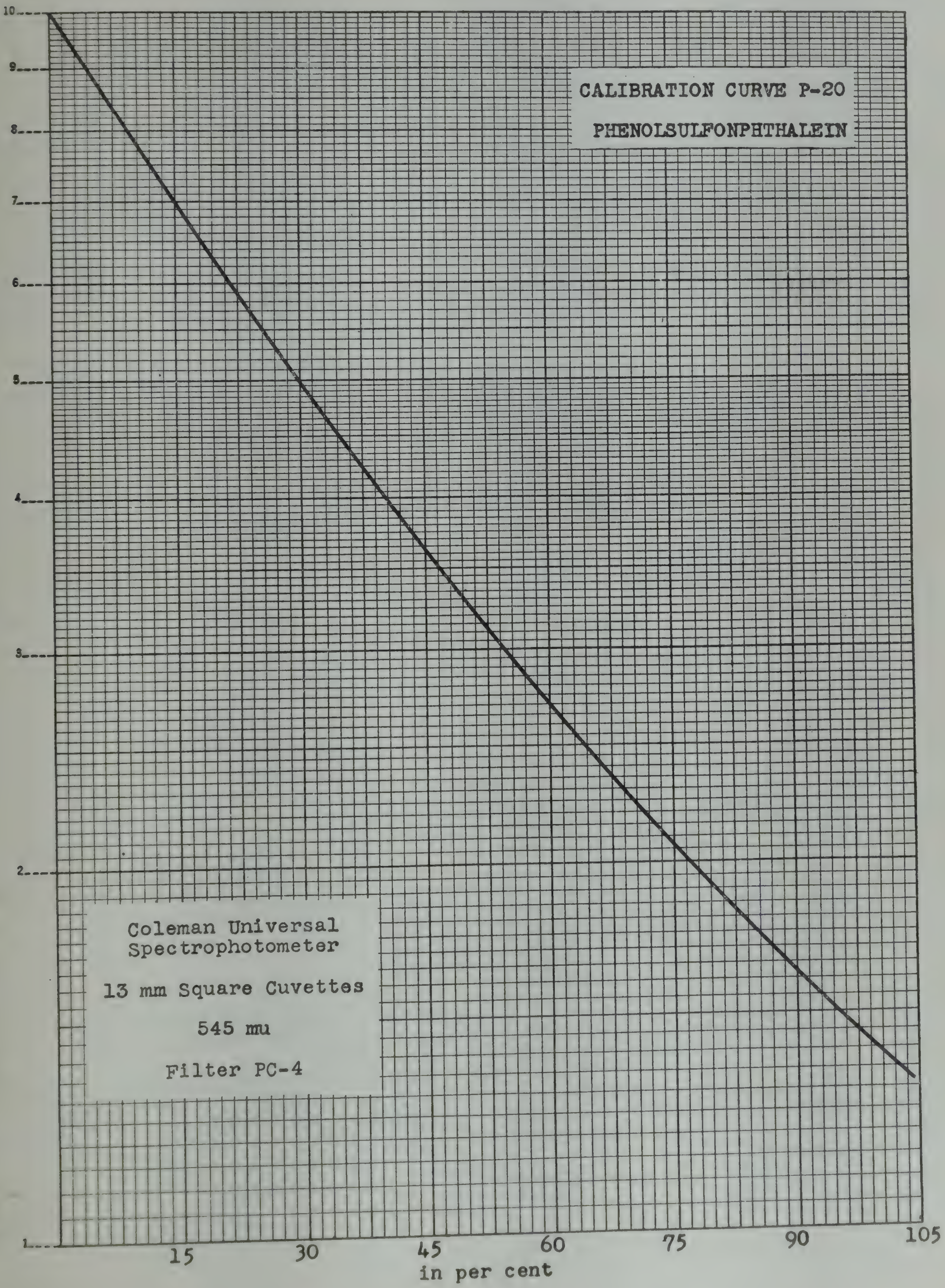
CALIBRATION CURVE P-20
PHENOLSULFONPHTHALEIN
Coleman Universal Spectrophotometer
13 mm Square Cuvettes
545 mu
Filter PC-4
10
9
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1
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Meter Reading	0	1	2	3	4	5	6	7	8	9
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PHOSPHATASE, ACID AND ALKALINE

A spectrophotometric adaptation based on the methods and modifications of Fiske and SubbaRow (1925), Bodansky (1933), Barringer and Woodard (1938), Roe and Whitmore (1938) and Woodard (1942)

PRINCIPLE

Serum is incubated with b-glycerophosphate substrates, buffered at pH 4.5 and/or 6.4 for acid phosphatase and at pH 8.6 for alkaline phosphatase. The amount of phosphorus liberated by the enzyme is determined as outlined in the method for inorganic phosphorus (Method P-36), the same analytical principles being involved. One Bodansky phosphatase unit is defined as the amount of phosphatase that liberates 1 mg of inorganic phosphorus from the b-glycerophosphate substrate in one hour under the conditions specified.

DETERMINATION IN SERUM

SAMPLE

Serum from blood withdrawn during post-absorptive state. Serum must be removed from clot as quickly as possible by centrifugation. There must be no trace of hemolysis. The analysis should not be unduly delayed, although the serum may be left in the refrigerator for several hours or in the frozen state for several days without any change in the phosphatase values.

TEST PROCEDURE

Special Apparatus

Glass-stoppered cylinders, 25 ml.
Ostwald-Folin pipettes accurately calibrated "to deliver" 1 ml.
Filter paper, double acid-washed, calcium and phosphorus-free, Whatman #44, 7 cm.
Graduated test tubes—10 ml in 0.1 ml; the 8 ml graduation must be accurately calibrated.
Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.

Reagents

1. **Sodium Chloride, 0.9% Aqueous.** Dissolve 9 gm sodium chloride (NaCl—Merck, Special for Biological Work) in distilled water and dilute to 1000 ml.
2. **Buffered Substrate, Acid, pH 4.5 (Barringer-Woodard).** In a 100 ml volumetric flask place 3 ml petroleum ether (J. T. Baker, Special, B. P. 20–40° C.) and 80 ml distilled water; add 500 mg sodium b-glycerophosphate ($C_3H_7O_3PO_3Na_2 \cdot 5H_2O$—Eastman #3532) and gently agitate the flask until solution is complete; dilute with distilled water to bring the aqueous level to the 100 ml mark and mix thoroughly. Adjust potentiometrically to pH 4.5 by the addition of 0.1 N hydrochloric acid. Preserve in refrigerator in Pyrex glass-stoppered bottle under a one inch layer of petroleum ether. Discard when the blank shows an appreciable increase.
3. **Buffered Substrate, Acid, pH 6.4 (Barringer-Woodard).** In a 100 ml volumetric flask place 3 ml petroleum ether (J. T. Baker, Special, B. P. 20–40° C.) and 80 ml distilled water; add 500 mg sodium b-glycerophosphate ($C_3H_7O_3PO_3Na_2 \cdot 5H_2O$—Eastman #3532) and gently agitate the flask until solution is complete; dilute with distilled water to bring the aqueous level to the 100 ml mark and mix thoroughly. Adjust potentiometrically to pH 6.4 by the addition of 0.1 N hydrochloric acid. Preserve in refrigerator in Pyrex glass-stoppered bottle under a one inch layer of petroleum ether. Discard when the blank shows an appreciable increase.
4. **Buffered Substrate, Alkaline, pH 8.6 (Bodansky).** In a 100 ml volumetric flask place 3 ml petroleum ether (J. T. Baker, Special, B. P. 20–40° C.) and 80 ml distilled water; add 500 mg sodium b-glycerophosphate ($C_3H_7O_3PO_3Na_2 \cdot 5H_2O$—Eastman #3532) and 424 mg sodium diethyl barbiturate ($C_8H_{11}O_3N_2Na$—Merck, U.S.P.) and gently agitate the flask until solution is complete; dilute with distilled water to bring the aqueous level to the 100 ml mark and mix thoroughly. Adjust potentiometrically to pH 8.6 by the addition of either 0.1 N hydrochloric acid or 0.1 N sodium hydroxide. Preserve in refrigerator in Pyrex glass-stoppered bottle under a one inch layer of petroleum ether. Discard when the blank shows an appreciable increase.
5. **Trichloracetic Acid, 30% Aqueous.** Dilute 30 ml concentrated trichloracetic acid (a) to 100 ml with distilled water. Preserve in glass-stoppered bottle. Stability indefinite.
    (a) Trichloracetic acid, concentrated; dissolve 100 gm crystalline trichloracetic acid (CCl_3-COOH—J. T. Baker, CP Analyzed) in distilled water and dilute to 100 ml. Preserve in Pyrex glass-stoppered bottle. Stability indefinite.
6. **Molybdate Reagent II (Fiske-SubbaRow).** In a 100 ml volumetric flask place 30 ml 10 N sulfuric acid (a); add 20 ml 12.5% ammonium molybdate (b) and dilute to volume with distilled water. Preserve in Pyrex glass-stoppered bottle.
    (a) Sulfuric acid, volumetric 10 N; slowly add 300 ml concentrated sulfuric acid (H_2SO_4—Grasselli, A.C.S.) to 750 ml distilled water; mix well and allow to cool; dilute

10 ml of this approximately 10 N solution to 100 ml; titrate against standard 1 N alkali using phenolphthalein as indicator; adjust accordingly and re-titrate—20 ml of a 1:10 dilution of 10 N acid require 20 ml of 1 N alkali. Preserve in Pyrex glass-stoppered bottle.

(b) Ammonium molybdate, 12.5% aqueous; dissolve 2.5 gm ammonium molybdate ($(NH_4)_6Mo_7O_{24} \cdot 4H_2O$—Mallinckrodt, AR) in distilled water and dilute to 20 ml. If larger quantities are prepared, preserve in ceresin-lined bottle. Discard when precipitate forms.

7. **Aminonaphthol Sulfonic Acid Reagent (Fiske-SubbaRow).** In a 100 ml mixing cylinder place 97.5 ml 15% sodium bisulfite (a) and 2.5 ml 20% sodium sulfite (b); add 250 mg recrystallized 1-amino-2-naphthol-4-sulfonic acid ($NH_2C_{10}H_5(OH)SO_3H$—Reagent Chemicals, Special) and shake until dissolved. With a high grade aminonaphthol sulfonic acid, solution is complete and rapid; with less suitable grades, it may be necessary to bring the acid into solution by the addition of a few more drops of the sulfite solution, carefully avoiding an excess. Preserve in a brown bottle away from light—stability approximately two weeks. The fresh solution should be almost colorless—on aging it acquires a yellow tinge; discard when the reagent blank shows an appreciable increase.
   (a) Sodium bisulfite, 15% aqueous; dissolve 15 gm sodium bisulfite ($NaHSO_3$—Merck, Reagent) in distilled water and dilute to 100 ml.
   (b) Sodium sulfite, 20% aqueous; dissolve 20 gm anhydrous sodium sulfite (Na_2SO_3—Baker & Adamson, A.C.S.) in distilled water and dilute to 100 ml.

Technic

Note: In doubtful cases where metastasizing carcinoma of the prostate is suspected, the acid phosphatase is determined at both pH 4.5 and 6.4—this is accomplished by including in the following procedure and treating in an identical manner an additional set of three tubes for the pH 6.4 buffered substrate (Reagent #3) and blanks.

For concentrations of liberated phosphorus in excess of 15 mg per 100 ml, dilute 1 ml serum to 2 or 4 ml with 0.9% sodium chloride (1) before proceeding with Step 5 and multiply the results by 2 or 4 respectively.

1. Label six 25 ml glass-stoppered cylinders "A," "B," "C," "D," "E," and "F."
2. In cylinder "A" (acid sample) place 9 ml acid buffered substrate (2).
3. In cylinder "B" (alkaline sample) place 9 ml alkaline buffered substrate (4).
4. Place both cylinders in water bath at 37° C. for 5 minutes.
5. To each cylinder add 1 ml serum from an Ostwald-Folin pipette—NOTE TIME.
6. Stopper, mix by inverting twice and place in water bath at 37° C. for EXACTLY 1 hour after the addition of the serum—the level of the fluid in the cylinders must be below the level of the water in the bath.
7. During the final 30 minutes of the above incubation period place in cylinders "C" (acid sample blank) and "D" (acid reagent blank) 9 ml acid buffered substrate (2).
8. Place in cylinders "E" (alkaline sample blank) and "F" (alkaline reagent blank) 9 ml alkaline buffered substrate (4).
9. Remove cylinders "A" and "B" from water bath at the end of EXACTLY 1 hour.
10. Immediately add 2 ml 30% trichloracetic acid (5) to all cylinders—start with cylinders "A" and "B."
11. To cylinders "C" and "E" slowly add 1 ml serum.
12. To cylinders "D" and "F" add 1 ml distilled water.
13. Stopper all cylinders, shake thoroughly and allow to stand for 5 minutes.
14. Filter through dry 7 cm double acid-washed filter papers into correspondingly marked test tubes.
15. Transfer 3 ml of each filtrate to a correspondingly marked 10 ml graduated test tube.
16. To each test tube add 0.5 ml molybdate reagent II (6) and mix.
17. To each test tube add 0.5 ml aminonaphthol sulfonic acid reagent (7) and mix.
18. Dilute to 8 ml with distilled water and mix.
19. Allow to stand in the dark for 10 minutes.
20. Measure the transmittancy of the acid sample "A" against the acid reagent blank "D" set at 100% transmission at a wave length of **660 $m\mu$** and multiply the value from the calibration table for inorganic phosphorus (Method P-36) by 1.2 to obtain the concentration of phosphorus in mg per 100 ml sample. If a calibration table has been prepared for phosphatase, the concentration is obtained directly. Record as result (a).
21. Measure the transmittancy of the acid sample blank "C" against the acid reagent blank "D" set at 100% transmission at a wave length of **660 $m\mu$** and obtain value as before. Record as result (b).
22. Measure the transmittancy of the alkaline sample "B" against the alkaline reagent blank "F" set at 100% transmission at a wave length of **660 $m\mu$** and obtain value as before. Record as result (c).
23. Measure the transmittancy of the alkaline sample blank "E" against the alkaline reagent blank "F" set at 100% transmission at a wave length of **660 $m\mu$** and obtain value as before. Record as result (d).

Calculation

Concentration of inorganic phosphorus in acid sample (a) in mg per 100 ml − Concentration of inorganic phosphorus in acid sample blank (b) in mg per 100 ml = Acid phosphatase in Bodansky units per 100 ml serum

Concentration of inorganic phosphorus in alkaline sample (c) in mg per 100 ml − Concentration of inorganic phosphorus in alkaline sample blank (d) in mg per 100 ml = Alkaline phosphatase in Bodansky units per 100 ml serum

CALIBRATION PROCEDURE

Note: The following calibration procedure is optional. The values in the calibration table for inorganic phosphorus (Method P-36), when multiplied by 1.2, are equivalent to the concentration of phosphorus in mg per 100 ml serum.

Special Apparatus

Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.

Graduated test tubes—10 ml in 0.1 ml; the 8 ml graduation must be accurately calibrated.

Reagents

6. **Molybdate Reagent II (Fiske-SubbaRow).** Same as for Test Procedure.
7. **Aminonaphthol Sulfonic Acid Reagent (Fiske-SubbaRow).** Same as for Test Procedure.
8. **Trichloracetic Acid, 5% Aqueous.** Dilute 5 ml concentrated trichloracetic acid (5a) to 100 ml with distilled water. Prepare fresh weekly.
9. **Phosphatase Standards.**
    (a) Phosphorus stock standard (1 ml contains 1 mg phosphorus). Dry recrystallized monopotassium phosphate (KH_2PO_4—Reagent Chemicals, Special) at 100° C. for one hour and cool in desiccator to constant weight; transfer 439.4 mg to a 100 ml volumetric flask; add approximately 50 ml distilled water and shake until dissolved; dilute to volume. Preserve with 5 ml chloroform.
    (b) Dilute standard (1 ml contains 0.01 mg phosphorus). In a 100 ml volumetric flask place 1 ml stock standard (9a) and dilute to volume with 5% trichloracetic acid (8). Prepare just before use.
    (c) Calibration standards. In a series of five accurately calibrated 10 ml volumetric flasks place respectively 0, 2.5, 5, 7.5 and 10 ml of the dilute standard (9b) and dilute to volume with 5% trichloracetic acid (8). These standards represent concentrations of phosphorus equivalent to 0, 3, 6, 9 and 12 mg per 100 ml sample. Prepare just before use.

Technic

1. Transfer 3 ml of each calibration standard (9c) to a correspondingly marked 10 ml graduated test tube.
2. Proceed exactly as outlined in the Test Procedure, Steps 16–19 inclusive.
3. Set the zero concentration standard, which should show no bluish color, at 100% transmission at a wave length of 660 mμ and record the transmittancy of the 3, 6, 9 and 12 mg per cent standards. The transmittancy of the 12 mg standard should be approximately 26%.
4. Plot the observed values on semi-log graph paper—the points fall on a straight line which passes through the origin (Calibration Curve P-25).
5. Prepare a calibration table from the T-C values obtained from the plotted curve.

PROCEDURE FOR USE WITH LINEAR-SCALE PHOTOELECTRIC COLORIMETERS (SHEARD-SANFORD TYPE)

TEST PROCEDURE

Exactly as outlined; set the reagent blanks at 100 and use a red filter.

CALIBRATION PROCEDURE

Exactly as outlined—use a red filter; plot the observed readings on semi-log graph paper—the points fall on a straight line passing through the origin.

PROCEDURE FOR USE WITH LOGARITHMIC-SCALE PHOTOELECTRIC COLORIMETERS (KLETT-SUMMERSON TYPE)

TEST PROCEDURE

Exactly as outlined; set the reagent blanks at 0 and use a red filter.

CALIBRATION PROCEDURE

Exactly as outlined—use a red filter; plot the observed readings on linear graph paper—the points fall on a straight line passing through the origin.

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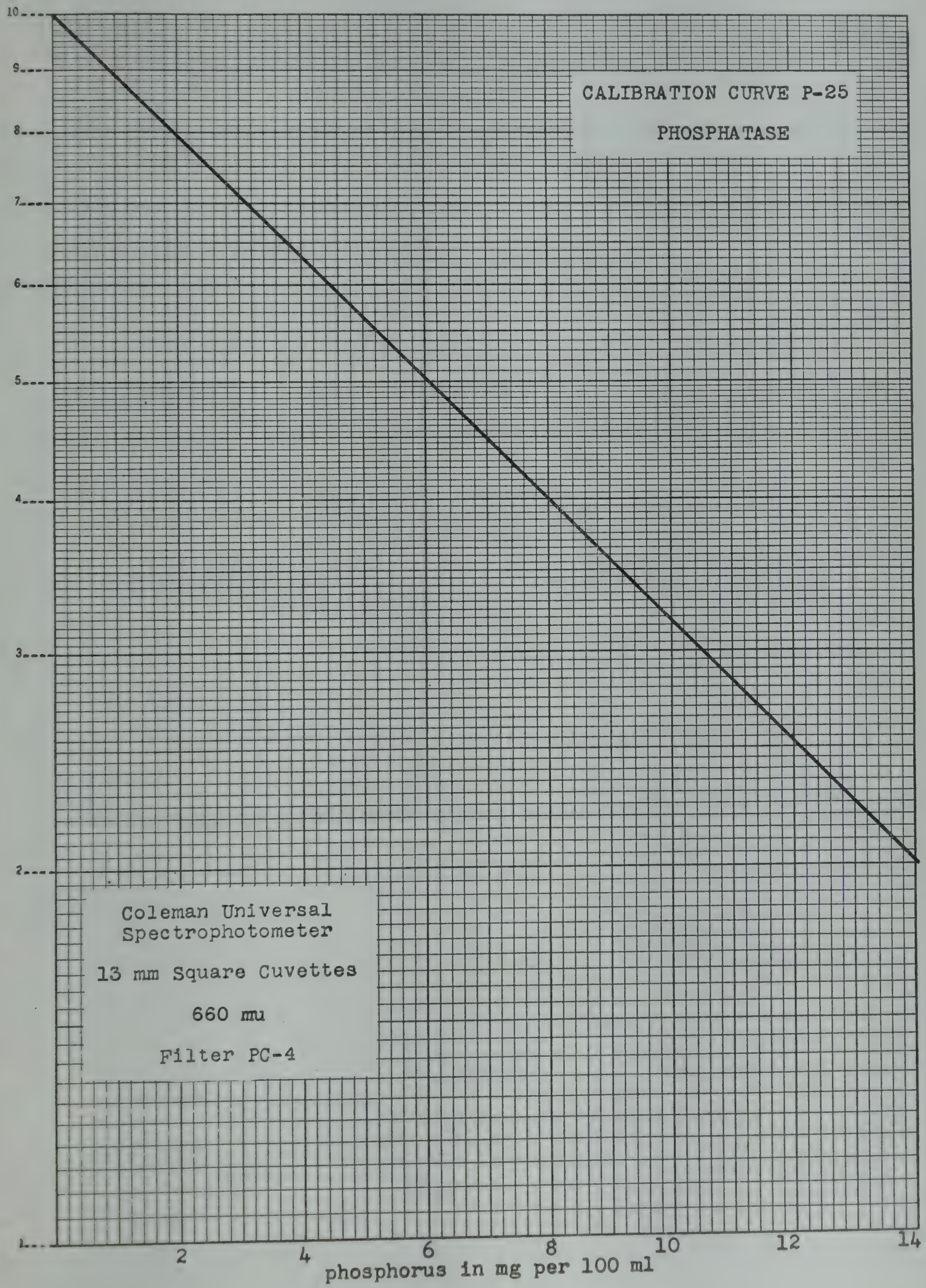
NOTES


CALIBRATION CURVE P-25
PHOSPHATASE
Coleman Universal Spectrophotometer
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phosphorus in mg per 100 ml

Meter Reading	0	1	2	3	4	5	6	7	8	9
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PHOSPHATASE, ACID

A spectrophotometric adaptation based on the methods and modifications of Folin and Ciocalteu (1927), King and Armstrong (1934) and Gutman and Gutman (1940)

PRINCIPLE

Serum is incubated with monophenyl phosphate substrate, buffered at pH 4.9. The amount of phenol liberated by the enzyme is determined by the blue color produced by reduction of a mixture of phosphomolybdic and phosphotungstic acids (Folin-Denis reaction). One Gutman phosphatase unit is defined as the amount of phosphatase that liberates 1 mg phenol from monophenyl phosphate substrate in one hour under the conditions specified.

DETERMINATION IN SERUM

SAMPLE

Serum from blood withdrawn during post-absorptive state. Serum must be removed from clot as quickly as possible by centrifugation. There must be no trace of hemolysis. The analysis should not be unduly delayed, although serum may be left in the refrigerator for several hours or in the frozen state for several days without any change in the phosphatase values.

TEST PROCEDURE

Special Apparatus

Glass-stoppered cylinders, 25 ml.

Ostwald-Folin pipettes accurately calibrated "to deliver" 0.5 ml.

Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.

Reagents

1. **Sodium Chloride, 0.9% Aqueous.** Dissolve 9 gm sodium chloride (NaCl—Merck, Special for Biological Work) in distilled water and dilute to 1000 ml.
2. **Buffered Substrate, Acid, pH 4.9 (Gutman).** Mix equal volumes of 0.01 M disodium monophenyl phosphate (a) and citrate buffer (b). Prepare just before use and check potentiometrically—if necessary adjust to pH 4.9 with 0.1 N hydrochloric acid or 0.1 N sodium hydroxide.
    - (a) Disodium monophenyl phosphate, 0.01 M; in a 500 ml volumetric flask place 1.09 gm disodium monophenyl phosphate ($C_6H_5OPO_3Na_2$—Paul-Lewis, Reagent); add about 400 ml distilled water; shake until dissolved and dilute to volume. Add a few drops of chloroform and preserve in refrigerator.
    - (b) Citrate buffer solution, pH 4.9 (Gutman); in a 500 ml volumetric flask place 21 gm crystalline citric acid ($C_6H_8O_7 \cdot H_2O$—Baker & Adamson, A.C.S.); add about 200 ml distilled water and shake until dissolved; add 188 ml 1 N sodium hydroxide (i); mix and dilute to volume with distilled water; adjust potentiometrically to pH 4.9 by the addition of either 0.1 N hydrochloric acid or 0.1 N sodium hydroxide. Add a few drops of chloroform and preserve in refrigerator.
        - (i) Sodium hydroxide, volumetric 1 N; prepare by diluting slightly more than the required amount of concentrated carbonate-free sodium hydroxide solution (see Appendix) and standardize against 1 N acid using phenolphthalein as indicator. Preserve in ceresin-lined bottle.
3. **Phenol Reagent, Dilute.** Dilute 1 volume concentrated phenol reagent (a) with 3 volumes distilled water shortly before use.
    - (a) Phenol reagent, concentrated (Folin-Ciocalteu); in a 1500 ml boiling flask with ground joint place 500 ml distilled water; add 100 gm sodium tungstate (Na_2WO_4-$2H_2O$—Mallinckrodt, Folin Special) and shake until dissolved; add 25 gm sodium molybdate ($Na_2MoO_4 \cdot 2H_2O$—Mallinckrodt, AR); add 200 ml distilled water washing down the neck of the flask and shake until solution is complete; add 50 ml 85% phosphoric acid (H_3PO_4—Baker & Adamson, A.C.S.) and mix thoroughly; add 100 ml concentrated hydrochloric acid (HCl—Baker & Adamson, A.C.S.) and mix thoroughly; fit a Liebig reflux condenser to the flask and boil gently for 10 hours (during this period the solution changes in color from a pale yellow to a deep green); remove the reflux condenser; add 150 gm lithium sulfate ($LiSO_4$—Baker & Adamson, Reagent) and 50 ml distilled water, washing down the neck of the flask; shake thoroughly until completely dissolved; add 10 drops bromine (Br—Baker & Adamson, A.C.S.) and boil for 15 minutes to remove the excess bromine; allow to cool to room temperature; dilute to 1000 ml with distilled water and filter through tightly packed glass wool into a brown glass-stoppered bottle. The reagent should show no tint of green and if kept well protected from dust will remain stable for months. A reagent which develops a green color in the

course of time may be repurified by the addition of a few drops of bromine and boiling as before.

4. **Sodium Carbonate, 20% Aqueous.** In a 1000 ml volumetric flask place 200 gm anhydrous sodium carbonate (Na_2CO_3—Baker & Adamson, A.C.S.); add 750 ml distilled water and shake until solution is complete; dilute to volume. Preserve in ceresin-lined bottle.

Technic

Note: For concentrations of liberated phenol in excess of 150 mg per 100 ml, dilute the serum with an equal volume of 0.9% sodium chloride (1) before proceeding with Step 5 and multiply the values obtained from the calibration table for phosphatase (Method P-30) by 2.

1. Label three 25 ml glass-stoppered cylinders "A," "B" and "C."
2. In cylinder "A" (sample) place 10 ml buffered substrate (2).
3. In cylinder "B" (sample blank) place 10 ml buffered substrate (2).
4. Place both cylinders in water bath at 37° C. for 5 minutes.
5. To cylinder "A" add 0.5 ml serum from an Ostwald-Folin pipette—NOTE TIME.
6. Stopper, mix by inverting twice and replace in water bath at 37° C. for EXACTLY 1 hour after the addition of the serum—the level of the fluid in the cylinders must be below the level of the water in the bath.
7. Remove both cylinders from water bath at the end of EXACTLY 1 hour.
8. Immediately add 4.5 ml dilute phenol reagent (3) to both cylinders, drop by drop and with constant agitation of the cylinders—start with cylinder "A."
9. To cylinder "B" add 0.5 ml serum and mix.
10. In cylinder "C" (reagent blank) place
    10.5 ml distilled water
    4.5 ml dilute phenol reagent (3) and mix.
11. Transfer the contents of the cylinders to correspondingly marked centrifuge tubes and centrifuge at 1500 rpm for 3 minutes.
12. Transfer 2 ml of the clear centrifugates to correspondingly marked 25 ml volumetric flasks.
13. To each flask add 10 ml distilled water and mix.
14. While constantly shaking the flasks add 2.5 ml 20% sodium carbonate (4).
15. Dilute to volume with distilled water and mix.
16. Place the flasks in water bath at 37° C. for 1 hour.
17. Allow to cool at room temperature for 20 minutes.
18. Measure the transmittancy of the sample "A" against the reagent blank "C" set at 100% transmission at a wave length of 660 mμ and obtain the concentration of phenol from the calibration table for phosphatase (Method P-30). Record as result (a).
19. Measure the transmittancy of the sample blank "B" against the reagent blank "C" set at 100% transmission at a wave length of 660 mμ and obtain the concentration as before. Record as result (b).

Calculation

Concentration of phenol in sample (a) in mg per 100 ml − Concentration of phenol in sample blank (b) in mg per 100 ml = Acid Phosphatase in Gutman units per 100 ml serum

CALIBRATION PROCEDURE

This calibration procedure need not be carried out. The values for phenol are obtained from the calibration table for phosphatase (Method P-30), the concentration of acid phosphatase being expressed in Gutman units per 100 ml serum.

PROCEDURE FOR USE WITH LINEAR-SCALE PHOTOELECTRIC COLORIMETERS (SHEARD-SANFORD TYPE)

TEST PROCEDURE

Exactly as outlined; set the reagent blank at 100 and use a red filter.

CALIBRATION PROCEDURE

Need not be performed. The values are obtained from the calibration table for phosphatase (Method P-30).

PROCEDURE FOR USE WITH LOGARITHMIC-SCALE PHOTOELECTRIC COLORIMETERS (KLETT-SUMMERSON TYPE)

TEST PROCEDURE

Exactly as outlined; set the reagent blank at 0 and use a red filter.

CALIBRATION PROCEDURE

Need not be performed. The values are obtained from the calibration table for phosphatase (Method P-30).

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PHOSPHATASE, ACID AND ALKALINE

A spectrophotometric adaptation based on the methods and modifications of
Folin and Ciocalteu (1927) and King and Armstrong (1934)

PRINCIPLE

Serum is incubated with monophenyl phosphate substrate, buffered at pH 5.0 for acid phosphatase and at pH 9.3 for alkaline phosphatase. The amount of phenol liberated by the enzyme is determined by the blue color produced by reduction of a mixture of phosphomolybdic and phosphotungstic acids (Folin-Denis reaction). One King-Armstrong phosphatase unit is defined as the amount of phosphatase that liberates 1 mg phenol from monophenyl phosphate substrate in thirty minutes under the conditions specified.

DETERMINATION IN SERUM

SAMPLE

Serum from blood withdrawn during post-absorptive state. Serum must be removed from clot as quickly as possible by centrifugation. There must be no trace of hemolysis. The analysis should not be unduly delayed, although serum may be left in the refrigerator for several hours or in the frozen state for several days without any change in the phosphatase values.

TEST PROCEDURE

Special Apparatus

Glass-stoppered cylinders, 25 ml.
Ostwald-Folin pipettes accurately calibrated "to deliver" 0.5 ml.
Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.

Reagents

1. **Sodium Chloride, 0.9% Aqueous.** Dissolve 9 gm sodium chloride (NaCl—Merck, Special for Biological Work) in distilled water and dilute to 1000 ml.
2. **Buffered Substrate, Acid, pH 5.0 (King-Armstrong).** In a 100 ml volumetric flask place 109 mg disodium monophenyl phosphate ($C_6H_5OPO_3$-Na_2—Paul-Lewis, Reagent); add 30 ml 0.2 N acetic acid (a) and shake until dissolved; dilute to volume with 0.2 N sodium acetate (b) and mix thoroughly. Adjust potentiometrically to pH 5.0 by the addition of either acetic acid or sodium acetate. Add 5 ml chloroform and preserve in refrigerator in resistant-glass bottle. Discard when the blank shows an appreciable increase.
    - (a) Acetic acid, volumetric 0.2 N; dilute 12 ml glacial acetic acid (CH_3COOH—Baker and Adamson, A.C.S.) to 1000 ml with distilled water and mix; titrate against standard 0.1 N alkali using phenolphthalein as indicator; adjust accordingly and re-titrate —10 ml of 0.2 N acid require 20 ml of 0.1 N alkali.
    - (b) Sodium acetate, 0.2 N; dissolve 27.22 gm crystalline sodium acetate ($NaC_2H_3O_2 \cdot 3H_2O$—Baker & Adamson, A.C.S.) in distilled water and dilute to 1000 ml.
3. **Buffered Substrate, Alkaline, pH 9.3 (King-Armstrong).** In a 100 ml volumetric flask place 109 mg disodium monophenyl phosphate (C_6H_5-OPO_3Na_2—Paul-Lewis, Reagent) and 1.03 gm sodium diethyl barbiturate ($C_8H_{11}O_3N_2Na$—Merck, U.S.P.); add 75 ml distilled water and shake until dissolved; dilute to volume. Adjust potentiometrically to pH 9.3 by the addition of either small amounts of sodium barbital or 0.1 N hydrochloric acid. Add 5 ml chloroform and preserve in refrigerator in resistant-glass bottle. Discard when the blank shows an appreciable increase.
4. **Phenol Reagent, Dilute.** Dilute 1 volume concentrated phenol reagent (a) with 3 volumes distilled water shortly before use.
    - (a) Phenol reagent, concentrated (Folin-Ciocalteu); in a 1500 ml boiling flask with ground joint place 500 ml distilled water; add 100 gm sodium tungstate ($Na_2WO_4 \cdot 2H_2O$—Mallinckrodt, Folin Special) and shake until dissolved; add 25 gm sodium molybdate ($Na_2MoO_4 \cdot 2H_2O$ — Mallinckrodt, AR); add 200 ml distilled water, washing down the neck of the flask and shake until solution is complete; add 50 ml 85% phosphoric acid (H_3PO_4—Baker & Adamson, A.C.S.) and mix thoroughly; add 100 ml concentrated hydrochloric acid (HCl—Baker & Adamson, A.C.S.) and mix thoroughly; fit a Liebig reflux condenser to the flask and boil gently for 10 hours (during this period the solution changes in color from a pale yellow to a deep green); remove the reflux condenser; add 150 gm lithium sulfate ($LiSO_4$—Baker & Adamson, Reagent) and 50 ml distilled water, washing down the neck of the flask; shake thoroughly until completely dissolved; add 10 drops bromine (Br—Baker & Adamson, A.C.S.) and boil for 15 minutes to remove the excess bromine; allow to cool to room temperature; dilute to 1000 ml with distilled water and filter through tightly packed glass wool into a brown glass-stoppered bottle. The reagent should show no tint of green and if kept well pro-

tected from dust will remain stable for months. A reagent which develops a green color in the course of time may be repurified by the addition of a few drops of bromine and boiling as before.

5. **Sodium Carbonate, 20% Aqueous.** In a 1000 ml volumetric flask place 200 gm anhydrous sodium carbonate (Na_2CO_3—Baker & Adamson, A.C.S.): add 750 ml distilled water and shake until solution is complete; dilute to volume. Preserve in ceresin-lined bottle.

Technic

Note: For concentrations of liberated phenol in excess of 150 mg per 100 ml, dilute the serum with an equal volume of 0.9% sodium chloride (1) before proceeding with Step 5 and multiply the values obtained from the calibration table by 2.

1. Label six 25 ml glass-stoppered cylinders "A," "B," "C," "D," "E" and "F."
2. In cylinder "A" (acid sample) place 10 ml acid buffered substrate (2).
3. In cylinder "B" (alkaline sample) place 10 ml alkaline buffered substrate (3).
4. Place both cylinders in water bath at 37.5° C. for 5 minutes.
5. To each cylinder add 0.5 ml serum from an Ostwald-Folin pipette—NOTE TIME.
6. Stopper, mix by inverting twice and place in water bath at 37.5° C. for EXACTLY 30 minutes after the addition of the serum—the level of the fluid in the cylinders must be below the level of the water in the bath.
7. During the final 15 minutes of the above incubation period place in cylinders "C" (acid sample blank) and "D" (acid reagent blank) 10 ml acid buffered substrate (2).
8. Place in cylinders "E" (alkaline sample blank) and "F" (alkaline reagent blank) 10 ml alkaline buffered substrate (3).
9. Remove cylinders "A" and "B" from water bath at the end of EXACTLY 30 minutes.
10. Immediately add 4.5 ml dilute phenol reagent (4) to all cylinders, drop by drop and with constant agitation of the cylinders—start with cylinders "A" and "B."
11. To cylinders "C" and "E" add 0.5 ml serum.
12. To cylinders "D" and "F" add 0.5 ml distilled water.
13. Stopper all cylinders and mix by inversion.
14. Transfer the contents of the cylinders to correspondingly marked centrifuge tubes and centrifuge at 1500 rpm for 3 minutes.
15. Transfer 2 ml of the clear centrifugates to correspondingly marked 25 ml volumetric flasks.
16. To each flask add 10 ml distilled water and mix.
17. While constantly shaking the flask add 2.5 ml 20% sodium carbonate (5), noting the time of addition to each flask and adding at fixed intervals in order that Steps 19–22 inclusive may be carried out at the exact time.
18. Dilute to volume with distilled water and mix.
19. EXACTLY 20 minutes after the addition of the sodium carbonate measure the transmittancy of the acid sample "A" against the acid reagent blank "D" set at 100% transmission at a wave length of **660** mμ and obtain the concentration of phenol from the calibration table. Record as result (a).
20. EXACTLY 20 minutes after the addition of the sodium carbonate measure the transmittancy of the acid sample blank "C" against the acid reagent blank "D" set at 100% transmission at a wave length of **660** mμ and obtain the concentration as before. Record as result (b).
21. EXACTLY 20 minutes after the addition of the sodium carbonate measure the transmittancy of the alkaline sample "B" against the alkaline reagent blank "F" set at 100% transmission at a wave length of **660** mμ and obtain the concentration as before. Record as result (c).
22. EXACTLY 20 minutes after the addition of the sodium carbonate measure the transmittancy of the alkaline sample blank "E" against the alkaline reagent blank "F" set at 100% transmission at a wave length of **660** mμ and obtain the concentration as before. Record as result (d).

Calculation

Concentration of phenol in acid sample (a) in mg per 100 ml − Concentration of phenol in acid sample blank (b) in mg per 100 ml = Acid Phosphatase in King-Armstrong units per 100 ml serum

Concentration of phenol in alkaline sample (c) in mg per 100 ml − Concentration of phenol in alkaline sample blank (d) in mg per 100 ml = Alkaline Phosphatase in King-Armstrong units per 100 ml serum

CALIBRATION PROCEDURE

Special Apparatus

Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.

Reagents

4. **Phenol Reagent, Dilute.** Same as for Test Procedure.
5. **Sodium Carbonate, 20% Aqueous.** Same as for Test Procedure.
6. **Hydrochloric Acid, Volumetric 0.1 N.** Add 10 ml concentrated hydrochloric acid (HCl—Baker & Adamson, A.C.S.) to 1000 ml distilled water and mix; standardize against 0.1 N alkali using phenolphthalein as indicator.

7. **Phosphatase Standards**
   (a) Phenol stock standard (1 ml contains 1 mg phenol). In a 1000 ml volumetric flask place slightly more than 1 gm crystalline phenol (C_6H_5OH—Baker & Adamson, Acid Carbolic, Reagent, Loose Crystals); add about 800 ml 0.1 N hydrochloric acid (6), shake until dissolved and dilute to volume with same. In a 250 ml glass-stoppered Erlenmeyer flask place 25 ml of the phenol solution; add 50 ml 0.1 N sodium hydroxide and heat to 65° C.; add 25 ml 0.1 N iodine and mix; stopper the flask and allow to stand at room temperature for 45 minutes; add 5 ml concentrated hydrochloric acid and titrate the excess iodine with 0.1 N sodium thiosulfate using starch as indicator—1 ml of 0.1 N iodine is equivalent to 1.567 mg phenol; adjust accordingly and re-titrate—the excess iodine after treatment of 25 ml phenol standard as outlined should require 9.05 ml sodium thiosulfate (i.e., 15.95 ml iodine combine with 25 mg phenol). Preserve in refrigerator. Stability indefinite.
   (b) Dilute standard (1 ml contains 0.1 mg phenol). In a 100 ml volumetric flask place 10 ml stock standard (7a) and dilute to volume with distilled water. Although this solution is stable for approximately 3 months in the refrigerator it is preferably prepared just before use.
   (c) Calibration standards. In a series of seven accurately calibrated 50 ml volumetric flasks place respectively 0, 1, 2, 4, 6, 8 and 10 ml of the dilute standard (7b) and dilute to volume with distilled water. These standards represent concentrations of phenol equivalent to 0, 15, 30, 60, 90, 120 and 150 mg per 100 ml sample. Prepare just before use.

Technic

1. Transfer 5 ml of each calibration standard (7c) to a correspondingly marked 25 ml volumetric flask.
2. To each flask add about 10 ml distilled water and mix.
3. Add 4.5 ml dilute phenol reagent (4), drop by drop and with constant agitation of the flask.
4. Proceed exactly as outlined in the Test Procedure, Steps 17–18 inclusive.
5. EXACTLY 20 minutes after the addition of the sodium carbonate set the zero concentration standard at 100% transmission at a wave length of 660 mμ and record the transmittancy of the 15, 30, 60, 90, 120 and 150 mg per cent standards. The transmittancy of the 150 mg standard should be approximately 27%.
6. Plot the observed values on semi-log graph paper—the points fall on a straight line which passes through the origin (Calibration Curve P-30).
7. Prepare a calibration table from the T-C values obtained from the plotted curve.

PROCEDURE FOR USE WITH LINEAR-SCALE PHOTOELECTRIC COLORIMETERS (SHEARD-SANFORD TYPE)

TEST PROCEDURE

Exactly as outlined; set the reagent blanks at 100 and use a red filter.

CALIBRATION PROCEDURE

Exactly as outlined—use a red filter; plot the observed readings on semi-log graph paper—the points fall on a straight line passing through the origin.

PROCEDURE FOR USE WITH LOGARITHMIC-SCALE PHOTOELECTRIC COLORIMETERS (KLETT-SUMMERSON TYPE)

TEST PROCEDURE

Exactly as outlined; set the reagent blanks at 0 and use a red filter.

CALIBRATION PROCEDURE

Exactly as outlined—use a red filter; plot the observed readings on linear graph paper—the points fall on a straight line passing through the origin.

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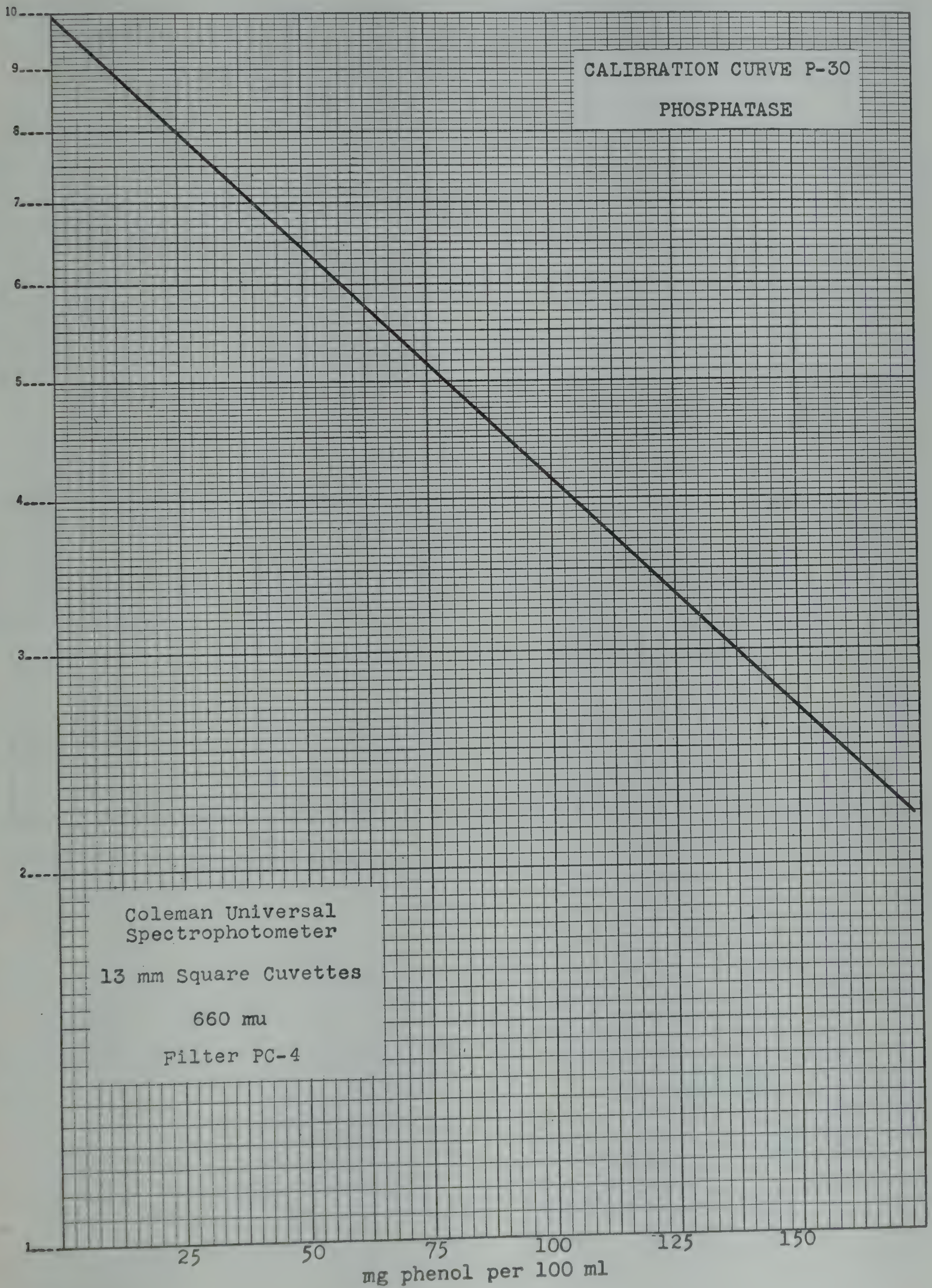
NOTES


CALIBRATION CURVE P-30
PHOSPHATASE
Coleman Universal Spectrophotometer
13 mm Square Cuvettes
660 mu
Filter PC-4
10
9
8
7
6
5
4
3
2
1
25
50
75
100
125
150
mg phenol per 100 ml

Meter Reading	0	1	2	3	4	5	6	7	8	9
0										
10										
20										
30										
40										
50										
60										
70										
80										
90										

PHOSPHATASE, ACID AND ALKALINE

A spectrophotometric adaptation based on the methods and modifications of Fiske and SubbaRow (1925) and Shinowara, Jones and Reinhart (1942)

PRINCIPLE

Serum is incubated with b-glycerophosphate substrate, buffered at pH 5.0 for acid phosphatase and at pH 10.9 for alkaline phosphatase. The amount of phosphorus liberated by the enzyme is determined as outlined in the method for inorganic phosphorus (Method P-36), the same analytical principles being involved. One Shinowara-Jones-Reinhart phosphatase unit is defined as the amount of phosphatase that liberates 1 mg of inorganic phosphorus from the b-glycerophosphate substrate in one hour under the conditions specified.

DETERMINATION IN SERUM

SAMPLE

Serum from blood withdrawn during post-absorptive state. Serum must be removed from clot as quickly as possible by centrifugation. There must be no trace of hemolysis. The analysis should not be unduly delayed, although the serum may be left in the refrigerator for several hours or in the frozen state for several days without any change in the phosphatase values.

TEST PROCEDURE

Special Apparatus

Glass-stoppered cylinders, 25 ml.

Ostwald-Folin pipettes accurately calibrated "to deliver" 1 ml.

Filter paper, double acid-washed, calcium and phosphorus-free, Whatman #44, 7 cm.

Graduated test tubes—10 ml in 0.1 ml; the 8 ml graduation must be accurately calibrated.

Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.

Reagents

1. **Sodium Chloride 0.9% Aqueous.** Dissolve 9 gm sodium chloride (NaCl—Merck, Special for Biological Work) in distilled water and dilute to 1000 ml.
2. **Buffered Substrate, Acid, pH 5.0 (Shinowara-Jones-Reinhart).** In a 100 ml volumetric flask place 3 ml petroleum ether (J. T. Baker, Special, B. P. 20–40° C.), 50 ml glycerophosphate buffer solution (a) and 5 ml 1 N acetic acid (b); dilute with distilled water to bring the aqueous level to to the 100 ml mark and mix thoroughly. Adjust potentiometrically to pH 5.0 by the addition of either 1 N acetic acid or glycerophosphate buffer solution. Preserve in refrigerator in Pyrex glass-stoppered bottle under a one inch layer of petroleum ether.
    (a) Glycerophosphate buffer solution (Shinowara-Jones-Reinhart); in a 200 ml volumetric flask place 3 ml petroleum ether (J. T. Baker, Special, B. P. 20–40° C.) and 160 ml distilled water; add 2 gm sodium b-glycerophosphate ($C_3H_7O_3PO_3Na_2 \cdot 5H_2O$—Eastman #3532) and 1.7 gm sodium diethyl barbiturate ($C_8H_{11}O_3N_2Na$—Merck, U.S.P.); shake until dissolved and dilute with distilled water to bring the aqueous level to the 200 ml mark and mix thoroughly. Preserve in refrigerator in Pyrex glass-stoppered bottle under a one inch layer of petroleum ether.
    (b) Acetic acid, volumetric 1 N; dilute 60 ml glacial acetic acid (CH_3COOH—Baker & Adamson, A.C.S.) to 1000 ml with distilled water and mix; standardize against 1 N alkali using phenolphthalein as indicator.
3. **Buffered Substrate, Alkaline, pH 10.9 (Shinowara-Jones-Reinhart).** In a 100 ml volumetric flask place 3 ml petroleum ether (J. T. Baker, Special, B. P. 20–40° C.), 50 ml glycerophosphate buffer solution (2a) and 2.8 ml 0.1 N sodium hydroxide (a); dilute with distilled water to bring the aqueous level to the 100 ml mark and mix thoroughly. Adjust potentiometrically to pH 10.9 by the addition of either 0.1 N sodium hydroxide or glycerophosphate buffer solution. Preserve in refrigerator in Pyrex glass-stoppered bottle under a one inch layer of petroleum ether.
    (a) Sodium hydroxide, volumetric 0.1 N; prepare by diluting slightly more than the required amount or concentrated carbonate-free sodium hydroxide solution (see Appendix) and standardize against 0.1 N acid using phenolphthalein as indicator. Preserve in ceresin-lined bottle.
4. **Trichloracetic Acid, 30% Aqueous.** Dilute 30 ml concentrated trichloracetic acid (a) to 100 ml with distilled water. Preserve in glass-stoppered bottle. Stability indefinite.
    (a) Trichloracetic acid, concentrated; dissolve 100 gm crystalline trichloracetic acid (CCl_3-$COOH$—J. T. Baker, CP Analyzed) in distilled water and dilute to 100 ml. Preserve in Pyrex glass-stoppered bottle. Stability indefinite.
5. **Molybdate Reagent II (Fiske-SubbaRow).** In a 100 ml volumetric flask place 30 ml 10 N sulfuric acid (a); add 20 ml 12.5% ammonium molybdate (b) and dilute to volume with distilled water. Preserve in Pyrex-glass-stoppered bottle.

(a) Sulfuric acid, volumetric 10 N; slowly add 300 ml concentrated sulfuric acid (H_2SO_4—Grasselli, A.C.S.) to 750 ml distilled water; mix well and allow to cool; dilute 10 ml of this approximately 10 N solution to 100 ml; titrate against standard 1 N alkali using phenolphthalein as indicator; adjust accordingly and re-titrate—20 ml of a 1:10 dilution of 10 N acid require 20 ml of 1 N alkali. Preserve in Pyrex glass-stoppered bottle.

(b) Ammonium molybdate, 12.5% aqueous; dissolve 2.5 gm ammonium molybdate ($(NH_4)_6Mo_7O_{24} \cdot 4H_2O$—Mallinckrodt, AR) in distilled water and dilute to 20 ml. If larger quantities are prepared, preserve in ceresin-lined bottle. Discard when precipitate forms.

6. **Aminonaphthol Sulfonic Acid Reagent (Fiske-SubbaRow).** In a 100 ml mixing cylinder place 97.5 ml 15% sodium bisulfite (a) and 2.5 ml 20% sodium sulfite (b); add 250 mg recrystallized 1-amino-2-naphthol-4-sulfonic acid (NH_2-$C_{10}H_5(OH)SO_3H$—Reagent Chemicals, Special) and shake until dissolved. With a high grade aminonaphthol sulfonic acid, solution is complete and rapid; with less suitable grades, it may be necessary to bring the acid into solution by the addition of a few more drops of the sulfite solution, carefully avoiding an excess. Preserve in a brown bottle away from light—stability approximately two weeks. The fresh solution should be almost colorless—on aging it acquires a yellow tinge; discard when the reagent blank shows an appreciable increase.

   (a) Sodium bisulfite, 15% aqueous; dissolve 15 gm sodium bisulfite ($NaHSO_3$—Merck, Reagent) in distilled water and dilute to 100 ml.

   (b) Sodium sulfite, 20% aqueous; dissolve 20 gm anhydrous sodium sulfite (Na_2SO_3, Baker & Adamson, A.C.S.) in distilled water and dilute to 100 ml.

Technic

Note: For concentrations of liberated phosphorus in excess of 15 mg per 100 ml, dilute 1 ml serum to 2 or 4 ml with 0.9% sodium chloride (1) before proceeding with Step 5 and multiply the results by 2 or 4 respectively.

1. Label six 25 ml glass-stoppered cylinders "A," "B," "C," "D," "E" and "F."
2. In cylinder "A" (acid sample) place 9 ml acid buffered substrate (2).
3. In cylinder "B" (alkaline sample) place 9 ml alkaline buffered substrate (3).
4. Place both cylinders in water bath at 38° C. for 5 minutes.
5. To each cylinder add 1 ml serum from an Ostwald-Folin pipette—NOTE TIME.
6. Stopper, mix by inverting twice and place in water bath at 38° C. for EXACTLY 1 hour after the addition of the serum—the level of the fluid in the cylinders must be below the level of the water in the bath.
7. During the final 30 minutes of the above incubation period place in cylinders "C" (acid sample blank) and "D" (acid reagent blank) 9 ml acid buffered substrate (2).
8. Place in cylinders "E" (alkaline sample blank) and "F" (alkaline reagent blank) 9 ml alkaline buffered substrate (3).
9. Remove cylinders "A" and "B" from water bath at the end of EXACTLY 1 hour.
10. Immediately add 2 ml 30% trichloracetic acid (4) to all cylinders—start with cylinders "A" and "B."
11. To cylinders "C" and "E" slowly add 1 ml serum.
12. To cylinders "D" and "F" add 1 ml distilled water.
13. Stopper all cylinders, shake thoroughly and allow to stand for 5 minutes.
14. Filter through dry 7 cm double acid-washed filter papers into correspondingly marked test tubes.
15. Transfer 3 ml of each filtrate to a correspondingly marked 10 ml graduated test tube.
16. To each tube add 0.5 ml molybdate reagent II (5) and mix.
17. To each tube add 0.5 ml aminonaphthol sulfonic acid reagent (6) and mix.
18. Dilute to 8 ml with distilled water and mix.
19. Allow to stand in the dark for 10 minutes.
20. Measure the transmittancy of the acid sample "A" against the acid reagent blank "D" set at 100% transmission at a wave length of 660 mμ and multiply the value from the calibration table for inorganic phosphorus (Method P-36) by 1.2 to obtain the concentration of phosphorus in mg per 100 ml sample. If a calibration table has been prepared for phosphatase, the concentration is obtained directly. Record as result (a).
21. Measure the transmittancy of the acid sample blank "C" against the acid reagent blank "D" set at 100% transmission at a wave length of 660 mμ and obtain value as before. Record as result (b).
22. Measure the transmittancy of the alkaline sample "B" against the alkaline reagent blank "F" set at 100% transmission at a wave length of 660 mμ and obtain value as before. Record as result (c).
23. Measure the transmittancy of the alkaline sample blank "E" against the alkaline reagent blank "F" set at 100% transmission at a wave length of 660 mμ and obtain value as before. Record as result (d).

Calculation

Concentration of inorganic phosphorus in acid sample (a) in mg per 100 ml − Concentration of inorganic phosphorus in acid sample blank (b) in mg per 100 ml = Acid phosphatase in S-J-R units per 100 ml serum

Concentration of inorganic phosphorus in alkaline sample (c) in mg per 100 ml − Concentration of inorganic phosphorus in alkaline sample blank (d) in mg per 100 ml = Alkaline phosphatase in S-J-R units per 100 ml serum

CALIBRATION PROCEDURE

Note: The following calibration procedure is optional. The values in the calibration table for inorganic phosphorus (Method P-36), when multiplied by 1.2, are equivalent to the concentration of phosphorus in mg per 100 ml serum.

Special Apparatus

Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.

Graduated test tubes—10 ml in 0.1 ml; the 8 ml graduation must be accurately calibrated.

Reagents

5. **Molybdate Reagent II (Fiske-SubbaRow).** Same as for Test Procedure.
6. **Aminonaphthol Sulfonic Acid Reagent (Fiske-SubbaRow).** Same as for Test Procedure.
7. **Trichloracetic Acid, 5% Aqueous.** Dilute 5 ml concentrated trichloracetic acid (4a) to 100 ml with distilled water. Prepare fresh weekly.
8. **Phosphatase Standards**
    - (a) Phosphorus stock standard (1 ml contains 1 mg phosphorus). Dry recrystallized monopotassium phosphate (KH_2PO_4—Reagent Chemicals, Special) at 100° C. for one hour and cool in desiccator to constant weight; transfer 439.4 mg to a 100 ml volumetric flask; add approximately 50 ml distilled water and shake until dissolved; dilute to volume. Preserve with 5 ml anhydrous chloroform.
    - (b) Dilute standard (1 ml contains 0.01 mg phosphorus). In a 100 ml volumetric flask place 1 ml stock standard (8a) and dilute to volume with 5% trichloracetic acid (7). Prepare just before use.
    - (c) Calibration standards. In a series of five accurately calibrated 10 ml volumetric flasks place respectively 0, 2.5, 5, 7.5 and 10 ml of the dilute standard (8b) and dilute to volume with 5% trichloracetic acid (7). These standards represent concentrations of phosphorus equivalent to 0, 3, 6, 9 and 12 mg per 100 ml sample. Prepare just before use.

Technic

1. Transfer 3 ml of each calibration standard (8c) to a correspondingly marked 10 ml graduated test tube.
2. Proceed exactly as outlined in the Test Procedure Steps 16–19 inclusive.
3. Set the zero concentration standard, which should show no bluish color, at 100% transmission at a wave length of **660** mμ and record the transmittancy of the 3, 6, 9 and 12 mg per cent standards. The transmittancy of the 12 mg standard should be approximately 26%.
4. Plot the observed values on semi-log graph paper—the points fall on a straight line which passes through the origin (Calibration Curve P-32).
5. Prepare a calibration table from the T-C values obtained from the plotted curve.

PROCEDURE FOR USE WITH LINEAR-SCALE PHOTOELECTRIC COLORIMETERS (SHEARD-SANFORD TYPE)

TEST PROCEDURE

Exactly as outlined; set the reagent blanks at 100 and use a red filter.

CALIBRATION PROCEDURE

Exactly as outlined—use a red filter; plot the observed readings on semi-log graph paper—the points fall on a straight line passing through the origin.

PROCEDURE FOR USE WITH LOGARITHMIC-SCALE PHOTOELECTRIC COLORIMETERS (KLETT-SUMMERSON TYPE)

TEST PROCEDURE

Exactly as outlined; set the reagent blanks at 0 and use a red filter.

CALIBRATION PROCEDURE

Exactly as outlined—use a red filter; plot the observed readings on linear graph paper—the points fall on a straight line passing through the origin.

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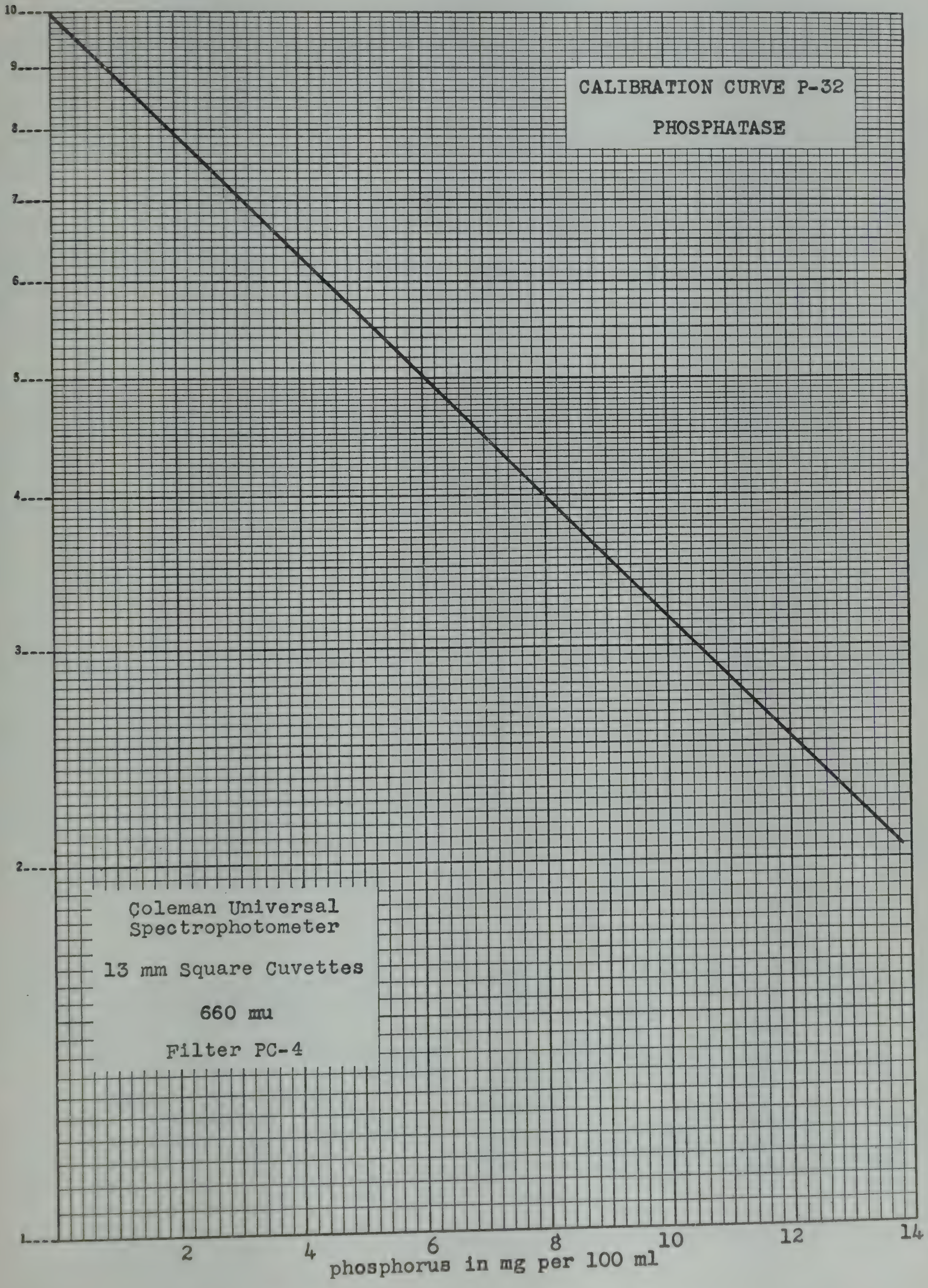
NOTES


CALIBRATION CURVE P-32
PHOSPHATASE
Coleman Universal Spectrophotometer
13 mm Square Cuvettes
660 mu
Filter PC-4
10
9
8
7
6
5
4
3
2
1
2
4
6
8
10
12
14
phosphorus in mg per 100 ml

Meter Reading	0	1	2	3	4	5	6	7	8	9
0										
10										
20										
30										
40										
50										
60										
70										
80										
90										

PHOSPHORUS, TOTAL ACID-SOLUBLE (ESTER)

A spectrophotometric adaptation based on the methods and modifications of Fiske and SubbaRow (1925), Walker and Huntsinger (1930) and Roe and Whitmore (1938)

PRINCIPLE

The organic matter in a protein-free trichloracetic acid filtrate is destroyed by means of sulfuric acid and hydrogen peroxide and the phosphorus in the digest is then determined as described for inorganic phosphorus (Method P-36), the same analytical principles being involved.

DETERMINATION IN WHOLE BLOOD, PLASMA OR SERUM

SAMPLE

Blood withdrawn during post-absorptive state. The serum may be preserved for several hours in the refrigerator or for a longer period by the addition of a drop of toluene. For total acid-soluble phosphorus determinations in plasma or whole blood, heparin should be used as an anticoagulant—oxalates inhibit color formation. Analyses preferably made on whole blood.

TEST PROCEDURE

Special Apparatus

Centrifuge tubes—15 ml, glass-stoppered.
Ostwald-Folin pipettes accurately calibrated "to deliver" 1 ml and 2 ml.
Digestion tubes, Pyrex, 25 × 200 mm.
Micro burner, Argand type.
Graduated test tubes—10 ml in 0.1 ml; the 8 ml graduation must be accurately calibrated.
Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.

Note: Glassware must be calcium and phosphorus-free. It should be thoroughly cleaned with dichromate-sulfuric acid solution (see Appendix), rinsed with distilled water and dried before use.

Reagents

1. **Trichloracetic Acid, 5% Aqueous.** Dilute 5 ml concentrated trichloracetic acid (a) to 100 ml with distilled water. Prepare fresh weekly.
    (a) Trichloracetic acid, concentrated; dissolve 100 gm crystalline trichloracetic acid (CCl_3-$COOH$—J. T. Baker, CP Analyzed) in distilled water and dilute to 100 ml. Preserve in Pyrex glass-stoppered bottle. Stability indefinite.
2. **Sulfuric Acid, Volumetric 10 N.** Slowly add 300 ml concentrated sulfuric acid (H_2SO_4—Grasselli, A.C.S.) to 750 ml distilled water; mix well and allow to cool; dilute 10 ml of this approximately 10 N solution to 100 ml; titrate against standard 1 N alkali using phenolphthalein as indicator; adjust accordingly and re-titrate—20 ml of a 1:10 dilution of 10 N acid require 20 ml of 1 N alkali. Preserve in Pyrex glass-stoppered bottle.
3. **Hydrogen Peroxide, 30%** (H_2O_2—Merck, Reagent Superoxol).
4. **Ammonium Molybdate, 2.5% Aqueous.** Dissolve 2.5 gm ammonium molybdate ($(NH_4)_6Mo_7O_{24} \cdot 4H_2O$—Mallinckrodt, AR) in distilled water and dilute to 100 ml. Preserve in ceresin-lined bottle. Discard when precipitate forms.
5. **Aminonaphthol Sulfonic Acid Reagent (Fiske-SubbaRow).** In a 100 ml mixing cylinder place 97.5 ml 15% sodium bisulfite (a) and 2.5 ml 20% sodium sulfite (b); add 250 mg recrystallized 1-amino-2-naphthol-4-sulfonic acid ($NH_2C_{10}H_5(OH)SO_3H$—Reagent Chemicals, Special) and shake until dissolved. With a high grade aminonaphthol sulfonic acid, solution is complete and rapid; with less suitable grades, it may be necessary to bring the acid into solution by the addition of a few more drops of the sulfite solution, carefully avoiding an excess. Preserve in a brown bottle away from light—stability approximately two weeks. The fresh solution should be almost colorless—on aging it acquires a yellow tinge; discard when the reagent blank shows an appreciable increase.
    (a) Sodium bisulfite, 15% aqueous; dissolve 15 gm sodium bisulfite ($NaHSO_3$—Merck, Reagent) in distilled water and dilute to 100 ml.
    (b) Sodium sulfite, 20% aqueous; dissolve 20 gm anhydrous sodium sulfite (Na_2SO_3—Baker & Adamson, A.C.S.) in distilled water and dilute to 100 ml.

Technic

For whole blood, follow technic as outlined; for plasma or serum, omit Step 3, take 3 ml filtrate for analysis and obtain the concentration of total acid-soluble phosphorus directly from the calibration table for inorganic phosphorus (Method P-36). If a calibration table has been prepared for total acid-soluble phosphorus, divide the values by 5.

1. In a 15 ml glass-stoppered centrifuge tube place
    9 ml 5% trichloracetic acid (1)
    1 ml sample from an Ostwald-Folin pipette, added drop by drop, with constant agitation of the tube.
2. Allow to stand for 5 minutes and centrifuge at 1500 rpm for 3 minutes.

3. In a 10 ml volumetric flask place 2 ml clear supernatant centrifugate and dilute to volume with distilled water; mix.
4. In a 25 × 200 mm Pyrex digestion tube place 3 ml diluted sample and one solid glass bead.
5. Heat with a very small flame and boil down to a volume of about 0.5 ml.
6. Add 0.5 ml 10 N sulfuric acid (2).
7. Heat gently over a small flame—as soon as charring occurs or fumes appear, reduce the flame until the digest barely boils and continue heating until no more charring occurs—superheating must be avoided and at no time should the flame touch the tube above the level of the fluid.
8. Allow to cool for 30 seconds and add 1 drop 30% hydrogen peroxide (3)—if the digest does not become colorless add another drop of peroxide and heat again—repeat this process until oxidation is complete.
9. Add 2 ml distilled water and heat to boiling momentarily.
10. Cool to room temperature under running tap water and quantitatively transfer the contents to a 10 ml graduated test tube (sample).
11. In another 10 ml graduated test tube (reagent blank) place
    0.5 ml 10 N sulfuric acid (2)
    5 ml distilled water.
12. To each tube add 0.5 ml 2.5% ammonium molybdate (4) and mix.
13. Add 0.5 ml aminonaphthol sulfonic acid reagent (5) and mix.
14. Dilute to 8 ml with distilled water; stopper with clean rubber stoppers and mix thoroughly by inversion.
15. Allow to stand in the dark for 10 minutes.
16. Measure the transmittancy of the sample against the reagent blank set at 100% transmission at a wave length of **660** mμ and multiply the value from the calibration table for inorganic phosphorus (Method P-36) by 5 to obtain the concentration of total acid-soluble phosphorus in mg per 100 ml sample. If a calibration table has been prepared for acid-soluble phosphorus, the concentration is obtained directly.

CALIBRATION PROCEDURE

This calibration procedure need not be carried out. A calibration curve (Calibration Curve P-35) may be constructed by substituting the values 10, 20, 30, 40 and 50 mg per 100 ml for the transmittancy readings recorded for the 2, 4, 6, 8 and 10 mg per 100 ml concentrations in the calibration curve for inorganic phosphorus.

PROCEDURE FOR USE WITH LINEAR-SCALE PHOTOELECTRIC COLORIMETERS (SHEARD-SANFORD TYPE)

TEST PROCEDURE

Exactly as outlined; set the reagent blank at 100 and use a red filter.

CALIBRATION PROCEDURE

Need not be performed. The values are obtained from the calibration table for inorganic phosphorus.

PROCEDURE FOR USE WITH LOGARITHMIC-SCALE PHOTOELECTRIC COLORIMETERS (KLETT-SUMMERSON TYPE)

TEST PROCEDURE

Exactly as outlined; set the reagent blank at 0 and use a red filter.

CALIBRATION PROCEDURE

Need not be performed. The values are obtained from the calibration table for inorganic phosphorus.

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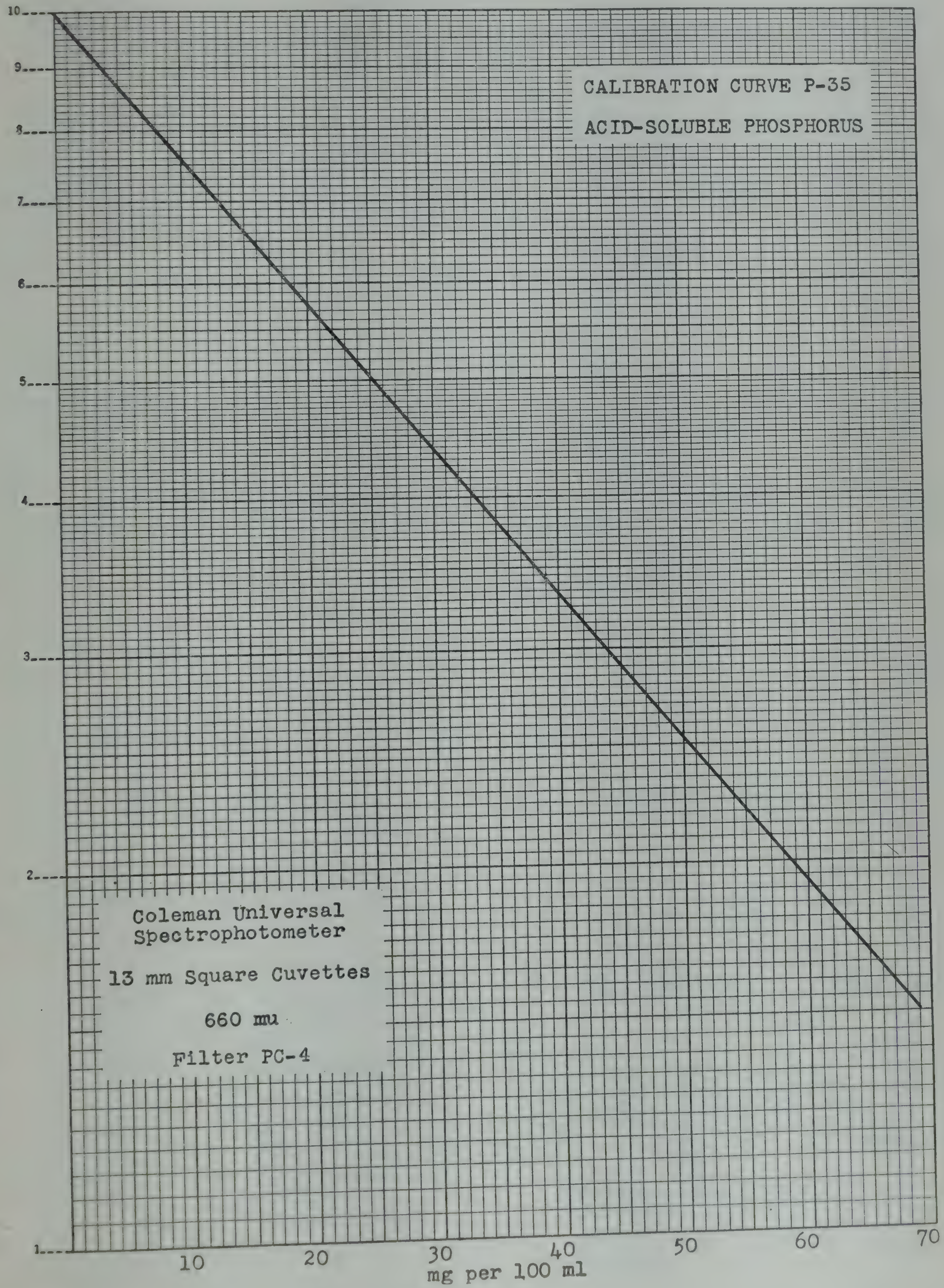
CALIBRATION CURVE P-35
ACID-SOLUBLE PHOSPHORUS
Coleman Universal Spectrophotometer
13 mm Square Cuvettes
660 mu
Filter PC-4
10
9
8
7
6
5
4
3
2
1
10
20
30
40
50
60
70
mg per 100 ml

Meter Reading	0	1	2	3	4	5	6	7	8	9
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10										
20										
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70										
80										
90										

PHOSPHORUS, INORGANIC

A spectrophotometric adaptation based on the methods and modifications of Fiske and SubbaRow (1925) and Roe and Whitmore (1938)

PRINCIPLE

Phosphorus, in an acid solution, is treated with ammonium molybdate to form a phosphomolybdate complex, which is then reduced by 1-amino-2-naphthol-4-sulfonic acid to form "molybdenum blue" (Fiske and SubbaRow reaction).

DETERMINATION IN WHOLE BLOOD, PLASMA OR SERUM

SAMPLE

Blood withdrawn during post-absorptive state. Serum must be removed from clot as quickly as possible by centrifugation. There must be no trace of hemolysis. The serum may be preserved for several hours in the refrigerator or for a longer period by the addition of a drop of toluene. The analysis should not be unduly delayed. For inorganic phosphorus determinations in plasma or whole blood, heparin should be used as an anticoagulant—oxalates inhibit color formation. Analyses preferably made on serum.

TEST PROCEDURE

Special Apparatus

Centrifuge tubes—15 ml, glass-stoppered.

Ostwald-Folin pipettes accurately calibrated "to deliver" 1 ml.

Filter paper, double acid-washed, calcium and phosphorus-free, Whatman #44, 7 cm (required only if filtrates instead of centrifugates are employed).

Graduated test tubes—10 ml in 0.1 ml; the 8 ml graduation must be accurately calibrated.

Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.

Note: Glassware must be calcium- and phosphorus-free. It should be thoroughly cleaned with dichromate-sulfuric acid solution (see Appendix), rinsed with distilled water and dried before use.

Reagents

1. **Trichloracetic Acid, 5% Aqueous.** Dilute 5 ml concentrated trichloracetic acid (a) to 100 ml with distilled water. Prepare fresh weekly.
   (a) Trichloracetic acid, concentrated; dissolve 100 gm crystalline trichloracetic acid (CCl_3-COOH—J. T. Baker, CP Analyzed) in distilled water and dilute to 100 ml. Preserve in Pyrex glass-stoppered bottle. Stability indefinite.
2. **Molybdate Reagent II (Fiske-SubbaRow).** In a 100 ml volumetric flask place 30 ml 10 N sulfuric acid (a); add 20 ml 12.5% ammonium molybdate (b) and dilute to volume with distilled water. Preserve in Pyrex glass-stoppered bottle.
   (a) Sulfuric acid, volumetric 10 N; slowly add 300 ml concentrated sulfuric acid (H_2SO_4—Grasselli, A.C.S.) to 750 ml distilled water; mix well and allow to cool; dilute 10 ml of this approximately 10 N solution to 100 ml; titrate against standard 1 N alkali using phenolphthalein as indicator; adjust accordingly and re-titrate—20 ml of a 1:10 dilution of 10 N acid require 20 ml of 1 N alkali. Preserve in Pyrex glass-stoppered bottle.
   (b) Ammonium molybdate, 12.5% aqueous; dissolve 2.5 gm ammonium molybdate ($(NH_4)_6Mo_7O_{24}\cdot 4H_2O$—Mallinckrodt, AR) in distilled water and dilute to 20 ml. If larger quantities are prepared, preserve in ceresin-lined bottle. Discard when precipitate forms.
3. **Aminonaphthol Sulfonic Acid Reagent (Fiske-SubbaRow).** In a 100 ml mixing cylinder place 97.5 ml 15% sodium bisulfite (a) and 2.5 ml 20% sodium sulfite (b); add 250 mg recrystallized 1-amino-2-naphthol-4-sulfonic acid ($NH_2C_{10}H_5$-$(OH)SO_3H$—Reagent Chemicals, Special) and shake until dissolved. With a high grade aminonaphthol sulfonic acid, solution is complete and rapid; with less suitable grades, it may be necessary to bring the acid into solution by the addition of a few more drops of the sulfite solution, carefully avoiding an excess. Preserve in a brown bottle away from light—stability approximately two weeks. The fresh solution should be almost colorless—on aging it acquires a yellow tinge; discard when the reagent blank shows an appreciable increase.
   (a) Sodium bisulfite, 15% aqueous; dissolve 15 gm sodium bisulfite ($NaHSO_3$—Merck, Reagent) in distilled water and dilute to 100 ml.
   (b) Sodium sulfite, 20% aqueous; dissolve 20 gm anhydrous sodium sulfite (Na_2SO_3—Baker & Adamson, A.C.S.) in distilled water and dilute to 100 ml.

Technic

Note: If the analysis is to be carried out on an aliquot of the trichloracetic acid centrifugate (or filtrate) as reserved in the calcium determination (Method C-10, Step 4), omit the following Steps 1–4 inclusive.

1. In a 15 ml glass-stoppered centrifuge tube (sample) place
   9 ml 5% trichloracetic acid (1)
   1 ml sample from an Ostwald-Folin pipette, added drop by drop, with constant agitation of the tube.

2. In another 15 ml glass-stoppered centrifuge tube (reagent blank) place
   9 ml 5% trichloracetic acid (1)
   1 ml distilled water.
3. Stopper both tubes and mix thoroughly.
4. Allow to stand for 5 minutes and centrifuge at 1500 rpm for 3 minutes (filtrates may be employed but the contents of *both* tubes must be filtered through dry 7 cm double acid-washed, calcium- and phosphorus-free papers into dry tubes). RESERVE PORTION FOR CALCIUM ANALYSIS (Method C-10).
5. In a 10 ml graduated test tube place 3 ml supernatant fluid (or filtrate) of sample.
6. In another 10 ml graduated test tube place 3 ml supernatant fluid (or filtrate) of reagent blank.
7. To each tube add 0.5 ml molybdate reagent II (2) and mix.
8. Add 0.5 ml aminonaphthol sulfonic acid reagent (3) and mix.
9. Dilute to 8 ml with distilled water; stopper with clean rubber stoppers and mix thoroughly by inversion.
10. Allow to stand in the dark for 10 minutes.
11. Measure the transmittancy of the sample against the reagent blank set at 100% transmission at a wave length of 660 mμ and obtain the concentration of phosphorus from the calibration table.

CALIBRATION PROCEDURE

Special Apparatus

Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.

Graduated test tubes—10 ml in 0.1 ml; the 8 ml graduation must be accurately calibrated.

Reagents

1. **Trichloracetic Acid, 5% Aqueous.** Same as for Test Procedure.
2. **Molybdate Reagent II (Fiske-SubbaRow).** Same as for Test Procedure.
3. **Aminonaphthol Sulfonic Acid Reagent (Fiske-SubbaRow).** Same as for Test Procedure.
4. **Phosphorus Standards**
   (a) Phosphorus stock standard, Roe-Whitmore (1 ml contains 1 mg phosphorus). Dry recrystallized monopotassium phosphate (KH_2PO_4—Reagent Chemicals, Special) at 100° C. for one hour and cool in desiccator to constant weight; transfer 439.4 mg to a 100 ml volumetric flask; add approximately 50 ml distilled water and shake until dissolved; dilute to volume with same. Preserve with 5 ml anhydrous chloroform.
   (b) Dilute standard (1 ml contains 0.01 mg phosphorus). In a 100 ml volumetric flask place 1 ml stock standard (4a) and dilute to volume with 5% trichloracetic acid (1). Prepare just before use.
   (c) Calibration standards. In a series of six accurately calibrated 10 ml volumetric flasks place respectively 0, 2, 4, 6, 8 and 10 ml of the dilute standard (4b) and dilute to volume with 5% trichloracetic acid (1). These standards represent concentrations of phosphorus equivalent to 0, 2, 4, 6, 8 and 10 mg per 100 ml sample. Prepare just before use.

Technic

1. Transfer 3 ml of each calibration standard (4c) to a correspondingly marked 10 ml graduated test tube.
2. Proceed exactly as outlined in the Test Procedure, Steps 7–10 inclusive.
3. Set the zero concentration standard, which should show no bluish color, at 100% transmission at a wave length of 660 mμ and record the transmittancy of the 2, 4, 6, 8 and 10 mg per cent standards. The transmittancy of the 10 mg standard should be approximately 26%.
4. Plot the observed values on semi-log graph paper —the points fall on a straight line which passes through the origin (Calibration Curve P-36).
5. Prepare a calibration table from the T-C values obtained from the plotted curve.

Method P-36b

DETERMINATION IN URINE

SAMPLE

Aliquot of a 24-hour specimen. Keep cold during collection.

TEST PROCEDURE

Special Apparatus

As listed under the Test Procedure for blood.

Reagents

1. **Trichloracetic Acid, 5% Aqueous.** Same as for blood.
2. **Molybdate Reagent II (Fiske-SubbaRow).** Same as for blood.
3. **Aminonaphthol Sulfonic Acid Reagent (Fiske-SubbaRow).** Same as for blood.
5. **Hydrochloric Acid, Concentrated** (HCl—Baker & Adamson, A.C.S.).

Technic

1. Place a 25 ml aliquot of the thoroughly mixed 24-hour specimen in a small Erlenmeyer flask and add 1–2 drops concentrated hydrochloric acid (5) to dissolve any precipitated phosphate.
2. Filter a small portion of the acidified urine.
3. In a 100 ml volumetric flask place 5 ml filtered urine and dilute to volume with distilled water.
4. In a 15 ml glass-stoppered centrifuge tube (sample) place
    - 9 ml 5% trichloracetic acid (1)
    - 1 ml diluted urine from an Ostwald-Folin pipette, added drop by drop.
5. Proceed exactly as outlined in the Test Procedure for blood, Steps 2–10 inclusive.
6. Measure the transmittancy of the sample against the reagent blank set at 100% transmission at a wave length of **660 mμ** and multiply the value from the calibration table for blood by 20 to obtain the concentration of phosphorus in mg per 100 ml urine.

Calculation

$$\frac{\text{Volume of 24-hour specimen}}{100} \times \text{Inorganic phosphorus in mg per 100 ml} = \text{Mg inorganic phosphorus excreted in 24 hours}$$

PROCEDURE FOR USE WITH LINEAR-SCALE PHOTOELECTRIC COLORIMETERS (SHEARD-SANFORD TYPE)

TEST PROCEDURE

Exactly as outlined; set the reagent blank at 100 and use a red filter.

CALIBRATION PROCEDURE

Exactly as outlined—use a red filter; plot the observed readings on semi-log graph paper—the points fall on a straight line passing through the origin.

PROCEDURE FOR USE WITH LOGARITHMIC-SCALE PHOTOELECTRIC COLORIMETERS (KLETT-SUMMERSON TYPE)

TEST PROCEDURE

Exactly as outlined; set the reagent blank at 0 and use a red filter.

CALIBRATION PROCEDURE

Exactly as outlined—use a red filter; plot the observed readings on linear graph paper—the points fall on a straight line passing through the origin.

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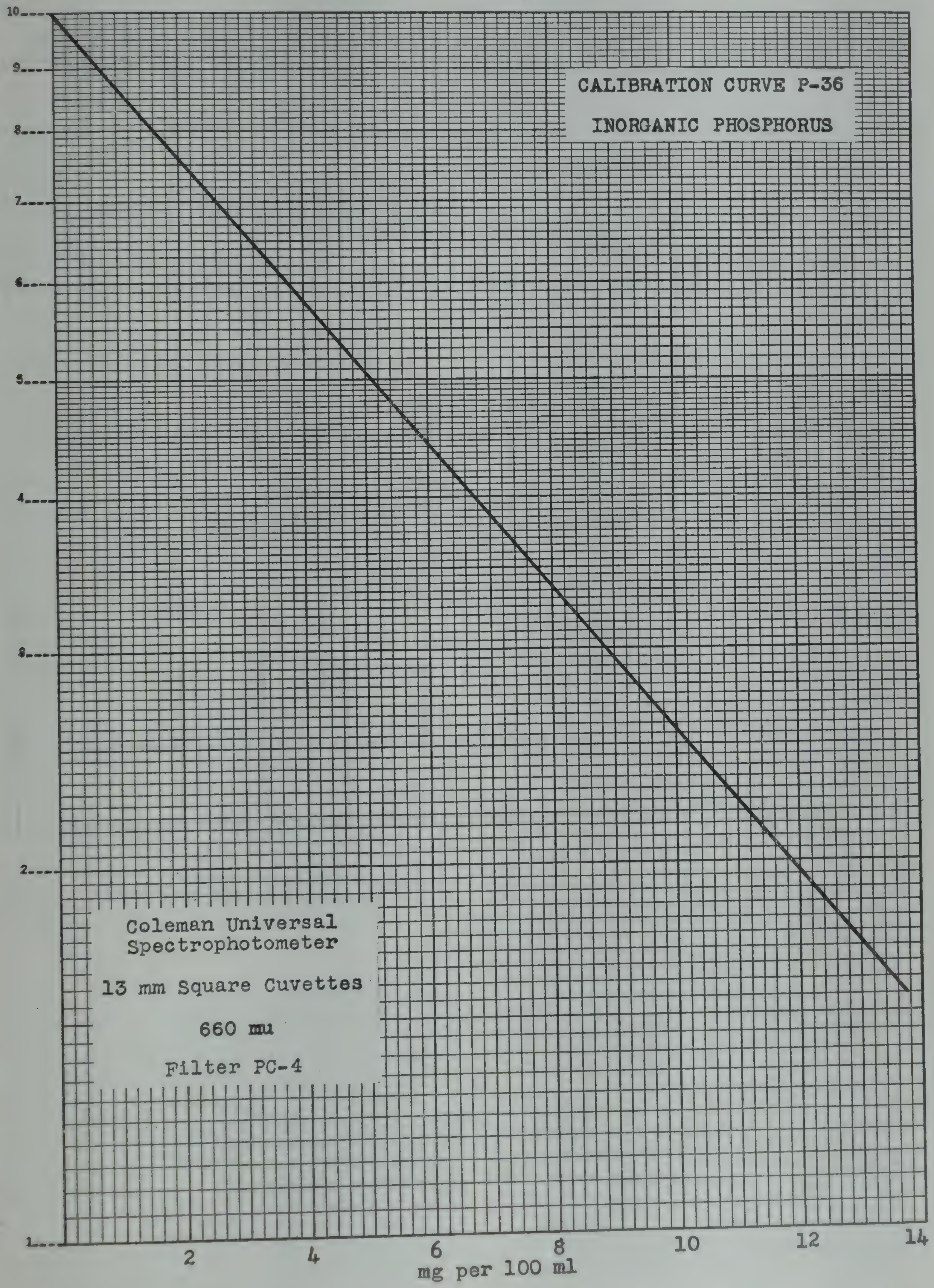
Roe, J. H., and Whitmore, E. R., "Clinico-Pathologic Application of Serum Phosphatase Determinations, with Special Reference to Lesions of the Bones," *Am. J. Clin. Path.*, **8**, 233 (1938).

NOTES

NOTES


CALIBRATION CURVE P-36
INORGANIC PHOSPHORUS
Coleman Universal Spectrophotometer
13 mm Square Cuvettes
660 mu
Filter PC-4
10
9
8
7
6
5
4
3
2
1
2
4
6
8
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12
14
mg per 100 ml

Meter Reading	0	1	2	3	4	5	6	7	8	9
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10										
20										
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50										
60										
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80										
90										

PHOSPHORUS, LIPOID

A spectrophotometric adaptation based on the methods and modifications of Fiske and SubbaRow (1925), Bloor (1928), Walker and Huntsinger (1930) and Roe and Whitmore (1938)

PRINCIPLE

The phospholipids are extracted from blood by a mixture of alcohol-ether. The organic matter in the extract is destroyed by means of sulfuric acid and hydrogen peroxide and the lipoid phosphorus in the digest is then determined as described for inorganic phosphorus (Method P-36), the same analytical principles being involved.

DETERMINATION IN WHOLE BLOOD, PLASMA OR SERUM

SAMPLE

Blood withdrawn during post-absorptive state. The serum may be preserved for several hours in the refrigerator or for a longer period by the addition of a drop of toluene. For lipoid phosphorus determinations in plasma or whole blood, heparin should be used as an anticoagulant—oxalates inhibit color formation. Analyses preferably made on plasma or serum.

TEST PROCEDURE

Special Apparatus

Ostwald-Folin pipettes accurately calibrated "to deliver" 1 ml.
Centrifuge tubes—15 ml, glass-stoppered.
Digestion tubes, Pyrex, 25 × 200 mm.
Micro burner, Argand type.
Graduated test tubes—10 ml in 0.1 ml; the 8 ml graduation must be accurately calibrated.
Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.

Note: Glassware must be calcium- and phosphorus-free. It should be thoroughly cleaned with dichromate-sulfuric acid solution (see Appendix), rinsed with distilled water and dried before use.

Reagents

1. **Alcohol:Ether Mixture, 3:1 (Bloor).** Mix three volumes 95% ethyl alcohol (C_2H_5OH—U.S. Industrial Chemicals, U.S.P.) with one volume anhydrous ether ($(C_2H_5)_2O$—Mallinckrodt, AR). Preserve in tightly stoppered brown glass bottle.
2. **Sulfuric Acid, Volumetric 10 N.** Slowly add 300 ml concentrated sulfuric acid (H_2SO_4—Grasselli, A.C.S.) to 750 ml distilled water; mix well and allow to cool; dilute 10 ml of this approximately 10 N solution to 100 ml; titrate against standard 1 N alkali using phenolphthalein as indicator; adjust accordingly and re-titrate—20 ml of a 1:10 dilution of 10 N acid require 20 ml of 1 N alkali. Preserve in Pyrex glass-stoppered bottle.
3. **Hydrogen Peroxide, 30%** (H_2O_2—Merck, Reagent Superoxol).
4. **Ammonium Molybdate, 2.5% Aqueous.** Dissolve 2.5 gm ammonium molybdate ($(NH_4)_6Mo_7O_{24}\cdot 4H_2O$—Mallinckrodt, AR) in distilled water and dilute to 100 ml. Preserve in ceresin-lined bottle. Discard when precipitate forms.
5. **Aminonaphthol Sulfonic Acid Reagent (Fiske-SubbaRow).** In a 100 ml mixing cylinder place 97.5 ml 15% sodium bisulfite (a) and 2.5 ml 20% sodium sulfite (b); add 250 mg recrystallized 1-amino-2-naphthol-4-sulfonic acid ($NH_2C_{10}H_5(OH)SO_3H$—Reagent Chemicals, Special) and shake until dissolved. With a high grade aminonaphthol sulfonic acid, solution is complete and rapid; with less suitable grades, it may be necessary to bring the acid into solution by the addition of a few more drops of the sulfite solution, carefully avoiding an excess. Preserve in a brown bottle away from light—stability approximately two weeks. The fresh solution should be almost colorless—on aging it acquires a yellow tinge; discard when the reagent blank shows an appreciable increase.
    (a) Sodium bisulfite, 15% aqueous; dissolve 15 gm sodium bisulfite ($NaHSO_3$—Merck, Reagent) in distilled water and dilute to 100 ml.
    (b) Sodium sulfite, 20% aqueous; dissolve 20 gm anhydrous sodium sulfite (Na_2SO_3—Baker & Adamson, A.C.S.) in distilled water and dilute to 100 ml.

Technic

Note: For concentrations of lipoid phosphorus in excess of 35 mg per 100 ml, use 1 ml centrifugate in Step 6 and multiply the values from the calibration table for inorganic phosphorus (Method P-36) by 6. If a calibration table has been prepared for lipoid phosphorus, multiply the values by 2.

1. In a 20 ml volumetric flask place
    18 ml alcohol:ether mixture (1)
    1 ml sample from an Ostwald-Folin pipette, added drop by drop, with constant agitation of the flask; stopper and mix thoroughly.
2. Heat in boiling water until the contents of the flask boils.
3. Cool to room temperature by placing in a beaker half-filled with cold water.
4. Adjust volume to 20 ml with alcohol:ether mixture (1) and mix.
5. Transfer a thoroughly mixed portion of the contents of the flask to a 15 ml glass-stoppered centrifuge tube and centrifuge at 1500 rpm for 3 minutes.

6. In a 25 × 200 mm Pyrex digestion tube place 2 ml clear supernatant centrifugate and one solid glass bead.
7. Heat with a very small flame and boil down to a volume of about 0.5 ml.
8. Add 0.5 ml 10 N sulfuric acid (2).
9. Heat gently over a small flame—as soon as charring occurs or fumes appear, reduce the flame until the digest barely boils and continue heating until no more charring occurs—superheating must be avoided and at no time should the flame touch the tube above the level of the fluid.
10. Allow to cool for 30 seconds and add 1 drop 30% hydrogen peroxide (3)—if the digest does not become colorless add another drop of peroxide and heat again—repeat this process until oxidation is complete.
11. Add 2 ml distilled water and heat to boiling momentarily.
12. Cool to room temperature under running tap water and quantitatively transfer the contents to a 10 ml graduated test tube (sample).
13. In another 10 ml graduated test tube (reagent blank) place
    0.5 ml 10 N sulfuric acid (2)
    5 ml distilled water.
14. To each tube add 0.5 ml 2.5% ammonium molybdate (4) and mix.
15. Add 0.5 ml aminonaphthol sulfonic acid reagent (5) and mix.
16. Dilute to 8 ml with distilled water; stopper with clean rubber stoppers and mix thoroughly by inversion.
17. Allow to stand in the dark for 10 minutes.
18. Measure the transmittancy of the sample against the reagent blank set at 100% transmission at a wave length of **660** mμ and multiply the value from the calibration table for inorganic phosphorus (Method P-36) by 3 to obtain the concentration of lipoid phosphorus in mg per 100 ml sample. If a calibration table has been prepared for lipoid phosphorus, the concentration is obtained directly.

CALIBRATION PROCEDURE

This calibration procedure need not be carried out. A calibration curve (Calibration Curve P-37) may be constructed by substituting the values 6, 12, 18, 24 and 30 mg per 100 ml for the transmittancy readings recorded for the 2, 4, 6, 8 and 10 mg per 100 ml concentrations in the calibration curve for inorganic phosphorus.

CONVERSION FACTOR

Lipoid phosphorus in mg per 100 ml × 25 = Lecithin in mg per 100 ml

PROCEDURE FOR USE WITH LINEAR-SCALE PHOTOELECTRIC COLORIMETERS (SHEARD-SANFORD TYPE)

TEST PROCEDURE

Exactly as outlined; set the reagent blank at 100 and use a red filter.

CALIBRATION PROCEDURE

Need not be performed. The values are obtained from the calibration table for inorganic phosphorus.

PROCEDURE FOR USE WITH LOGARITHMIC-SCALE PHOTOELECTRIC COLORIMETERS (KLETT-SUMMERSON TYPE)

TEST PROCEDURE

Exactly as outlined; set the reagent blank at 0 and use a red filter.

CALIBRATION PROCEDURE

Need not be performed. The values are obtained from the calibration table for inorganic phosphorus.

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Walker, B. S., and Huntsinger, M. E., "The Phosphorus Partition in Normal Whole Blood," *J. Lab. Clin. Med.*, **16**, 247 (1930).
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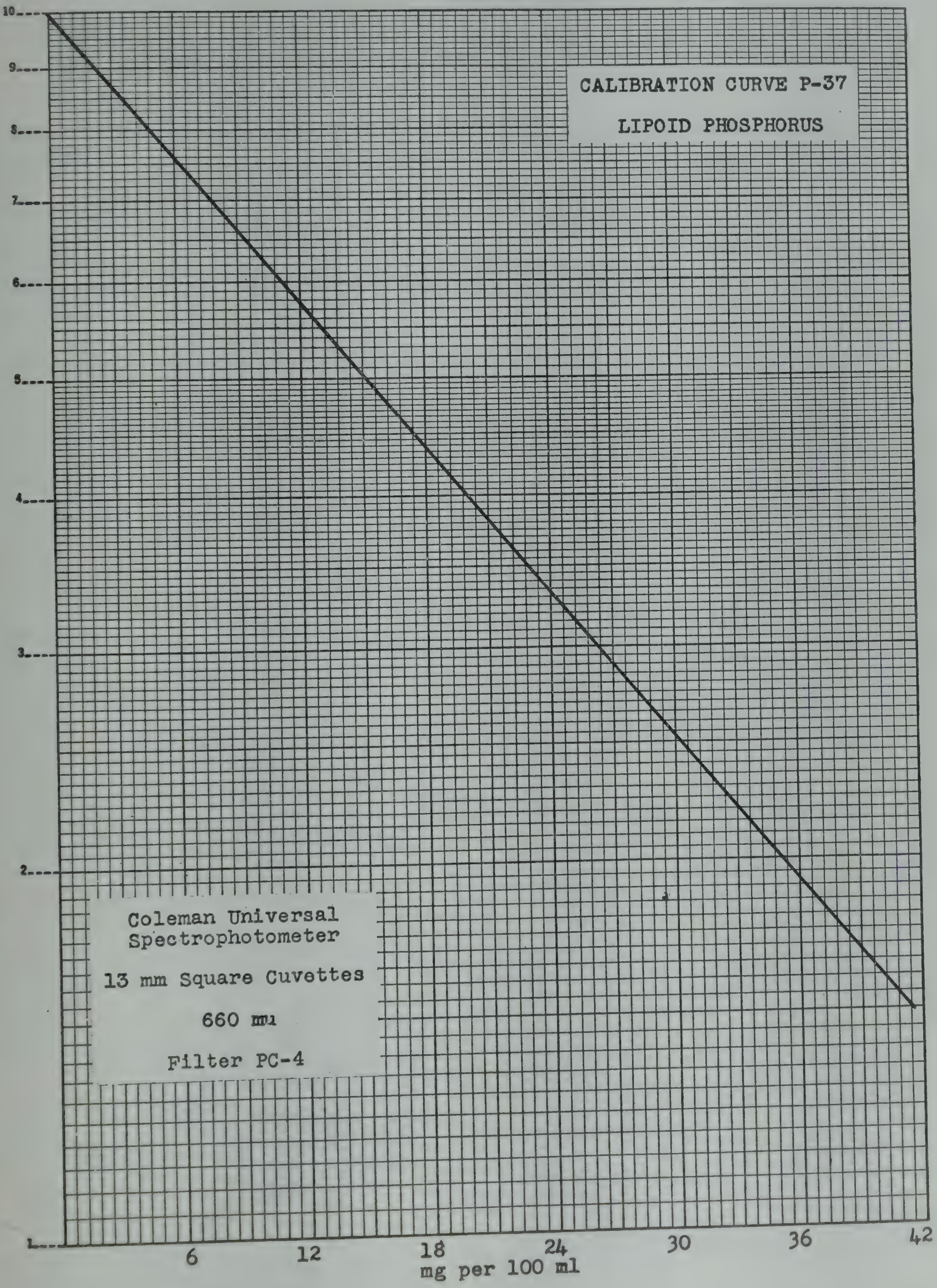
CALIBRATION CURVE P-37
LIPOID PHOSPHORUS
Coleman Universal Spectrophotometer
13 mm Square Cuvettes
660 mμ
Filter PC-4
10
9
8
7
6
5
4
3
2
1
6
12
18
24
30
36
42
mg per 100 ml

Meter Reading	0	1	2	3	4	5	6	7	8	9
0										
10										
20										
30										
40										
50										
60										
70										
80										
90										

PHOSPHORUS, TOTAL

A spectrophotometric adaptation based on the methods and modifications of Fiske and SubbaRow (1925), Walker and Huntsinger (1930) and Roe and Whitmore (1938)

PRINCIPLE

The organic matter in serum is destroyed by means of sulfuric acid and hydrogen peroxide and the phosphorus in the digest is then determined as described for inorganic phosphorus (Method P-36), the same analytical principles being involved.

DETERMINATION IN WHOLE BLOOD, PLASMA OR SERUM

SAMPLE

Blood withdrawn during post-absorptive state. The serum may be preserved for several hours in the refrigerator or for a longer period by the addition of a drop of toluene. For total phosphorus determinations in plasma or whole blood, heparin should be used as an anticoagulant—oxalates inhibit color formation. Analyses preferably made on whole blood.

TEST PROCEDURE

Special Apparatus

Micro pipettes accurately calibrated "to contain" 0.2 ml.

Digestion tubes, Pyrex, 25 × 200 mm.

Micro burner, Argand type.

Graduated test tubes—10 ml in 0.1 ml; the 8 ml graduation must be accurately calibrated.

Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.

Note: Glassware must be calcium- and phosphorus-free. It should be thoroughly cleaned with dichromate-sulfuric acid solution (see Appendix), rinsed with distilled water and dried before use.

Reagents

1. **Sodium Chloride, 0.9% Aqueous.** Dissolve 9 gm sodium chloride (NaCl—Merck, Special for Biological Work) in distilled water and dilute to 1000 ml.
2. **Sulfuric Acid, Volumetric 10 N.** Slowly add 300 ml concentrated sulfuric acid (H_2SO_4—Grasselli, A.C.S.) to 750 ml distilled water; mix well and allow to cool; dilute 10 ml of this approximately 10 N solution to 100 ml; titrate against standard 1 N alkali using phenolphthalein as indicator; adjust accordingly and re-titrate—20 ml of a 1:10 dilution of 10 N acid require 20 ml of 1 N alkali. Preserve in Pyrex glass-stoppered bottle.
3. **Hydrogen Peroxide, 30%** (H_2O_2—Merck, Reagent Superoxol).
4. **Ammonium Molybdate, 2.5% Aqueous.** Dissolve 2.5 gm ammonium molybdate ($(NH_4)_6Mo_7O_{24} \cdot 4H_2O$—Mallinckrodt, AR) in distilled water and dilute to 100 ml. Preserve in ceresin-lined bottle. Discard when precipitate forms.
5. **Aminonaphthol Sulfonic Acid Reagent (Fiske-SubbaRow).** In a 100 ml mixing cylinder place 97.5 ml 15% sodium bisulfite (a) and 2.5 ml 20% sodium sulfite (b); add 250 mg recrystallized 1-amino-2-naphthol-4-sulfonic acid ($NH_2C_{10}H_5(OH)SO_3H$—Reagent Chemicals, Special) and shake until dissolved. With a high grade aminonaphthol sulfonic acid, solution is complete and rapid; with less suitable grades, it may be necessary to bring the acid into solution by the addition of a few more drops of the sulfite solution, carefully avoiding an excess. Preserve in a brown bottle away from light—stability approximately two weeks. The fresh solution should be almost colorless—on aging it acquires a yellow tinge; discard when the reagent blank shows an appreciable increase.
   - (a) Sodium bisulfite, 15% aqueous; dissolve 15 gm sodium bisulfite ($NaHSO_3$—Merck, Reagent) in distilled water and dilute to 100 ml.
   - (b) Sodium sulfite, 20% aqueous; dissolve 20 gm anhydrous sodium sulfite (Na_2SO_3—Baker & Adamson, A.C.S.) in distilled water and dilute to 100 ml.

Technic

1. In a 20 ml volumetric flask place
   15 ml 0.9% sodium chloride (1)
   0.2 ml sample from a micro pipette, thoroughly rinsing out the pipette by drawing in and expelling some of the mixture several times; dilute to volume with sodium chloride.
2. In a 25 × 200 mm Pyrex digestion tube place 3 ml diluted sample and one solid glass bead.
3. Heat with a very small flame and boil down to a volume of about 0.5 ml.
4. Add 0.5 ml 10 N sulfuric acid (2).
5. Heat gently over a small flame—as soon as charring occurs or fumes appear, reduce the flame until the digest barely boils and continue heating until no more charring occurs—superheating must be avoided and at no time should the flame touch the tube above the level of the fluid.
6. Allow to cool for 30 seconds and add 1 drop 30% hydrogen peroxide (3)—if the digest does not become colorless add another drop of peroxide and heat again—repeat this process until oxidation is complete.

7. Add 2 ml distilled water and heat to boiling momentarily.
8. Cool to room temperature under running tap water and quantitatively transfer the contents to a 10 ml graduated test tube (sample).
9. In another 10 ml graduated test tube (reagent blank) place
   0.5 ml 10 N sulfuric acid (2)
   5 ml distilled water.
10. To each tube add 0.5 ml 2.5% ammonium molybdate (4) and mix.
11. Add 0.5 ml aminonaphthol sulfonic acid reagent (5) and mix.
12. Dilute to 8 ml with distilled water; stopper with clean rubber stoppers and mix thoroughly by inversion.
13. Allow to stand in the dark for 10 minutes.
14. Measure the transmittancy of the sample against the reagent blank set at 100% transmission at a wave length of **660** mμ and multiply the value from the calibration table for inorganic phosphorus (Method P-36) by 10 to obtain the concentration of total phosphorus in mg per 100 ml sample. If a calibration table has been prepared for total phosphorus, the concentration is obtained directly.

CALIBRATION PROCEDURE

This calibration procedure need not be carried out. A calibration curve (Calibration Curve P-38) may be constructed by substituting the values 20, 40, 60, 80 and 100 mg per 100 ml for the transmittancy readings recorded for the 2, 4, 6, 8 and 10 mg per 100 ml concentrations in the calibration curve for inorganic phosphorus.

Method P-38b

DETERMINATION IN URINE

SAMPLE

Aliquot of a 24-hour specimen. Keep cold during collection.

TEST PROCEDURE

Special Apparatus

As listed under the Test Procedure for blood.

Reagents

2. **Sulfuric Acid, Volumetric 10 N.** Same as for blood.
3. **Hydrogen Peroxide, 30%.** Same as for blood.
4. **Ammonium Molybdate, 2.5% Aqueous.** Same as for blood.
5. **Aminonaphthol Sulfonic Acid Reagent (Fiske-SubbaRow).** Same as for blood.
6. **Hydrochloric Acid, Concentrated** (HCl—Baker & Adamson, A.C.S.).

Technic

1. Place a 25 ml aliquot of the thoroughly mixed 24-hour specimen in a small Erlenmeyer flask and add 1–2 drops concentrated hydrochloric acid (6) to dissolve any precipitated phosphate.
2. Filter a small portion of the acidified urine.
3. In a 100 ml volumetric flask place 5 ml filtered urine and dilute to volume with distilled water.
4. In a 10 ml volumetric flask place 1 ml diluted urine and dilute to volume with distilled water; mix.
5. Proceed exactly as outlined in the Test Procedure for blood, Steps 2–13 inclusive.
6. Measure the transmittancy of the sample against the reagent blank set at 100% transmission at a wave length of **660** mμ and multiply the value from the calibration table for inorganic phosphorus in blood (Method P-36) by 20 to obtain the concentration of total phosphorus in mg per 100 ml urine. If a calibration table has been prepared for total phosphorus in blood multiply the value by 2.

Calculation

$$\frac{\text{Volume of 24-hour specimen}}{100} \times \text{Total phosphorus in mg per 100 ml} = \text{Mg total phosphorus excreted in 24 hours}$$

PROCEDURE FOR USE WITH LINEAR-SCALE PHOTOELECTRIC COLORIMETERS (SHEARD-SANFORD TYPE)

TEST PROCEDURE

Exactly as outlined; set the reagent blank at 100 and use a red filter.

CALIBRATION PROCEDURE

Need not be performed. The values are obtained from the calibration table for inorganic phosphorus.

PROCEDURE FOR USE WITH LOGARITHMIC-SCALE PHOTOELECTRIC COLORIMETERS (KLETT-SUMMERSON TYPE)

TEST PROCEDURE

Exactly as outlined; set the reagent blank at 0 and use a red filter.

CALIBRATION PROCEDURE

Need not be performed. The values are obtained from the calibration table for inorganic phosphorus.

BIBLIOGRAPHY

Fiske, C. H., and SubbaRow, Y., "The Colorimetric Determination of Phosphorus," *J. Biol. Chem.*, **66**, 375 (1925).

Walker, B. S., and Huntsinger, M. E., "The Phosphorus Partition in Normal Whole Blood," *J. Lab. Clin. Med.*, **16**, 247 (1930).

Roe, J. H., and Whitmore, E. R., "Clinico-Pathologic Application of Serum Phosphatase Determinations, with Special Reference to Lesions of the Bones," *Am. J. Clin. Path.*, **8**, 233 (1938).

NOTES

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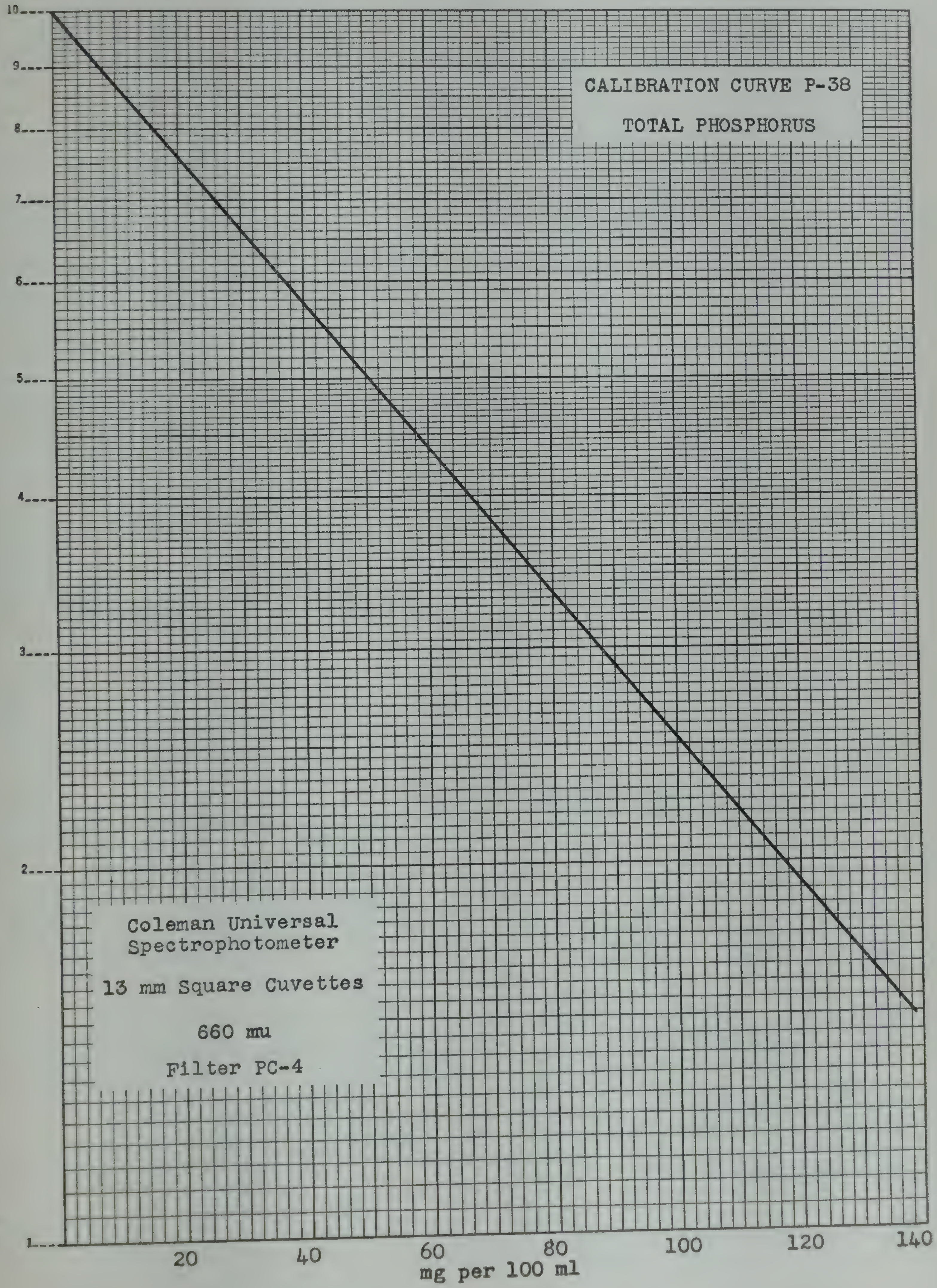
CALIBRATION CURVE P-38
TOTAL PHOSPHORUS
Coleman Universal Spectrophotometer
13 mm Square Cuvettes
660 mu
Filter PC-4
10
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6
5
4
3
2
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60
80
100
120
140
mg per 100 ml

Meter Reading	0	1	2	3	4	5	6	7	8	9
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10										
20										
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40										
50										
60										
70										
80										
90										

POTASSIUM

A spectrophotometric adaptation based on the methods and modifications of Kramer and Tisdall (1921), Jacobs and Hoffman (1931), Hoffman and Jacobs (1934) and Hoffman (1937; 1941)

PRINCIPLE

Potassium is precipitated as potassium sodium cobaltinitrite, the cobalt content being used as a measure of the potassium. Upon treatment of the cobalt-containing precipitate with choline chloride and potassium ferrocyanide, emerald green cobalt choline ferrocyanide is produced.

DETERMINATION IN SERUM

SAMPLE

Serum from blood withdrawn during post-absorptive state. Draw blood with a minimum of stasis, using a dry needle and syringe, and transfer to a dry centrifuge tube without spraying. Immediately (or at least within one-half hour) after clotting is complete (about 15 minutes at 37° C.), gently separate the clot from the walls of the tube with a thin glass rod or wire and centrifuge at once. Draw off serum completely with a capillary pipette and re-centrifuge. There must not be the slightest trace of hemolysis. Clear serum may be stored at least 48 hours in refrigerator.

TEST PROCEDURE

Special Apparatus

Centrifuge tubes with sharply pointed tips—Pyrex, 15 ml.
Ostwald-Folin pipettes accurately calibrated "to deliver" 1 ml.
Wash bottles, all-glass, 125 ml.
Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.

Note: Glassware must be thoroughly cleaned with hot dilute nitric acid, rinsed with distilled water and dried before use—dichromate-sulfuric acid cleaning solution should not be used. Precautions should be taken to prevent contamination of all glassware and reagents by ammonia vapor.

Reagents

1. **Sodium Acetate, Half-Saturated Aqueous.** Dilute saturated sodium acetate (a) with an equal volume of distilled water.
    (a) Sodium acetate, saturated aqueous; in an 8 ounce bottle place 130 gm finely pulverized crystalline sodium acetate ($NaC_2H_3O_2 \cdot 3H_2O$—Baker & Adamson, A.C.S.) and add 100 ml distilled water previously heated to 45° C.; shake thoroughly and allow to cool and settle. Use the clear supernatant fluid. Note: Complete saturation is essential.
2. **Sodium Cobaltinitrite Reagent (Kramer-Tisdall).** Place 62.5 ml cobalt nitrate solution (a) in a 500 ml Florence flask; under a fume hood, add 210 ml sodium nitrite solution (b)—an evolution of gas occurs; stopper flask with a 2-hole rubber stopper fitted with a long inlet tube which passes well below the surface of the liquid and a short outlet tube which is connected to a water pump; aerate the solution until there is no odor of nitrous oxide (about two hours); transfer to a brown glass-stoppered bottle. Store in refrigerator—when odor re-develops, aerate as above. Filter a small portion just before use. Stability approximately 3 months.
    (a) Cobalt nitrate solution; dissolve 25 gm cobalt nitrate ($Co(NO_3)_2 \cdot 6H_2O$—Mallinckrodt, A.C.S.) in 50 ml distilled water; add 12.5 ml glacial acetic acid (CH_3COOH—Baker & Adamson, A.C.S.); stir thoroughly.
    (b) Sodium nitrite solution; add 120 gm granular potassium-free sodium nitrite ($NaNO_2$—Mallinckrodt, A.C.S.) to 180 ml distilled water; stir until solution is complete.
3. **Wash Solution (Hoffman).** Add a small amount of potassium sodium cobaltinitrite ($K_2NaCo(NO_2)_6 \cdot H_2O$—Reagent Chemicals, Special) to 95 ml 10% ethyl alcohol (a) and shake until the solution is saturated; transfer to storage bottle without removing the excess solid. Just before use filter a portion through a Schleicher and Schuell #576 paper into an all-glass wash bottle.
    (a) Ethyl alcohol, 10% aqueous; dilute 10 ml 95% ethyl alcohol (C_2H_5OH—U.S. Industrial Chemicals, U.S.P.) to 95 ml with distilled water; mix and adjust to volume.
4. **Ethyl Alcohol, 70% Aqueous.** Dilute 370 ml 95% ethyl alcohol (C_2H_5OH—U.S. Industrial Chemicals, U.S.P.) to 500 ml with distilled water; mix and adjust to volume. Use in an all-glass wash bottle.
5. **Choline Chloride, 1% Aqueous.** Dissolve 1 gm crystalline choline chloride ($(CH_3)_3NClCH_2CH_4$-OH—Eastman #1122) in distilled water; dilute to 100 ml and filter.
6. **Potassium Ferrocyanide Solution.** Just before use dilute 1 ml saturated potassium ferrocyanide (a) to 25 ml with distilled water. Larger amounts should not be prepared and the solution remaining after use should be discarded.
    (a) Potassium ferrocyanide, saturated aqueous; place 30 ml distilled water in a 150 ml beaker; bring to a boil; add approxi-

mately 35 gm freshly pulverized potassium ferrocyanide ($K_4Fe(CN)_6 \cdot 3H_2O$—Baker & Adamson, A.C.S.); stir; transfer while hot to a small brown glass-stoppered bottle; allow to cool and use supernatant fluid. Discard when a blank determination shows that some of the ferrocyanide has been oxidized to ferricyanide.

Technic

Note: For concentrations of potassium in excess of 24 mg per 100 ml, use 0.5 ml serum in Step 1 and multiply the values obtained from the calibration table by 2.

1. In a 15 ml Pyrex centrifuge tube (sample) place
   1 ml serum, delivered from an Ostwald-Folin pipette
   1 ml half-saturated sodium acetate (1) and mix
   1 ml freshly filtered sodium cobaltinitrite reagent (2), added slowly, drop by drop, while constantly agitating the tube.
2. Allow to stand at room temperature for 45 minutes —protect from ammonia vapor.
3. Wash down the sides of the tube with 2 ml distilled water.
4. Centrifuge at 2000 rpm for 15 minutes.
5. Decant the supernatant fluid and place the tube in an inverted position on a piece of filter paper for 15 minutes; wipe the mouth of the tube with a clean cloth.
6. Wash with 2–3 ml wash solution (3) from an all-glass wash bottle.
7. Centrifuge again for 15 minutes and drain as before.
8. Using a glass rod, stir up the precipitate in 2–3 ml 70% ethyl alcohol (4), washing off the rod with another 1–2 ml alcohol.
9. Centrifuge again for 5 minutes and drain as before.
10. Repeat the washing with 70% ethyl alcohol, centrifuge for 5 minutes and drain as before.
11. Add 2 ml distilled water without stirring up the precipitate.
12. Place the tube in a boiling water bath—in about 15 minutes most of the precipitate will be decomposed and dissolved.
13. With the aid of a stirring rod, break up any undissolved precipitate, scouring the sides of the tube.
14. Remove the stirring rod while washing off with 2–3 ml distilled water.
15. Replace the tube in boiling water bath for 5 minutes—solution should be complete, except for traces of protein, which may be ignored.
16. Allow to cool to room temperature.
17. In another Pyrex centrifuge tube (reagent blank) place
    1 ml distilled water.
18. To each tube add 1 ml 1% choline chloride (5) and mix.
19. Add 1 ml potassium ferrocyanide solution (6)—these reagents must be added in the order named; a green color appears immediately.
20. Quantitatively transfer the contents of each tube to a correspondingly marked 20 ml volumetric flask; dilute to volume with distilled water and mix thoroughly by inversion.
21. Measure the transmittancy of the sample against the reagent blank set at 100% transmission at a wave length of **420** mμ and obtain the concentration of potassium from the calibration table.

CALIBRATION PROCEDURE

Special Apparatus

Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.
Centrifuge tubes with sharply pointed tips—Pyrex, 15 ml.
Wash bottles, all-glass, 125 ml.

Reagents

1. **Sodium Acetate, Half-Saturated Aqueous.** Same as for Test Procedure.
2. **Sodium Cobaltinitrite Reagent (Kramer-Tisdall).** Same as for Test Procedure.
3. **Wash Solution (Hoffman).** Same as for Test Procedure.
4. **Ethyl Alcohol, 70% Aqueous.** Same as for Test Procedure.
5. **Choline Chloride, 1% Aqueous.** Same as for Test Procedure.
6. **Potassium Ferrocyanide Solution.** Same as for Test Procedure.
7. **Potassium Standards**
   (a) Potassium stock standard (1 ml contains 3 mg potassium). Dry recrystallized potassium sulfate (K_2SO_4—Reagent Chemicals, Special) at 100° C. for one hour and cool in desiccator to constant weight; transfer 668.5 mg to a 100 ml volumetric flask; add approximately 50 ml distilled water and shake until dissolved; dilute to volume with same.
   (b) Dilute standard (1 ml contains 0.3 mg potassium). In a 100 ml volumetric flask place 10 ml stock standard (7a) and dilute to volume with distilled water. Prepare just before use.
   (c) Calibration standards. In a series of five accurately calibrated 10 ml volumetric flasks place respectively 0, 2, 4, 6 and 8 ml of the dilute standard (7b) and dilute to volume with distilled water. These standards represent concentrations of potassium equivalent to 0, 6, 12, 18 and 24 mg per 100 ml sample. Prepare just before use.

Technic

1. Transfer 1 ml of each calibration standard (7c) to a correspondingly marked Pyrex centrifuge tube.
2. To each tube add
    1 ml half-saturated sodium acetate (1) and mix
    1 ml freshly filtered sodium cobaltinitrite reagent (2), added slowly, drop by drop, while constantly agitating the tube.
3. Proceed exactly as outlined in the Test Procedure, Steps 2–20 inclusive.
4. Set the zero concentration standard at 100% transmission at a wave length of **420 mμ** and record the transmittancy of the 6, 12, 18 and 24 mg per cent standards. The transmittancy of the 24 mg standard should be approximately 28%.
5. Plot the observed values on semi-log graph paper—the points fall on a smooth curve which passes through the origin (Calibration Curve P-40).
6. Prepare a calibration table from the T-C values obtained from the plotted curve.

PROCEDURE FOR USE WITH LINEAR-SCALE PHOTOELECTRIC COLORIMETERS (SHEARD-SANFORD TYPE)

TEST PROCEDURE

Exactly as outlined; set the reagent blank at 100 and use a blue filter.

CALIBRATION PROCEDURE

Exactly as outlined—use a blue filter; plot the observed readings on semi-log graph paper—the points fall on a smooth curve passing through the origin.

PROCEDURE FOR USE WITH LOGARITHMIC-SCALE PHOTOELECTRIC COLORIMETERS (KLETT-SUMMERSON TYPE)

TEST PROCEDURE

Exactly as outlined; set the reagent blank at 0 and use a blue filter.

CALIBRATION PROCEDURE

Exactly as outlined—use a blue filter; plot the observed readings on linear graph paper—the points fall on a smooth curve passing through the origin.

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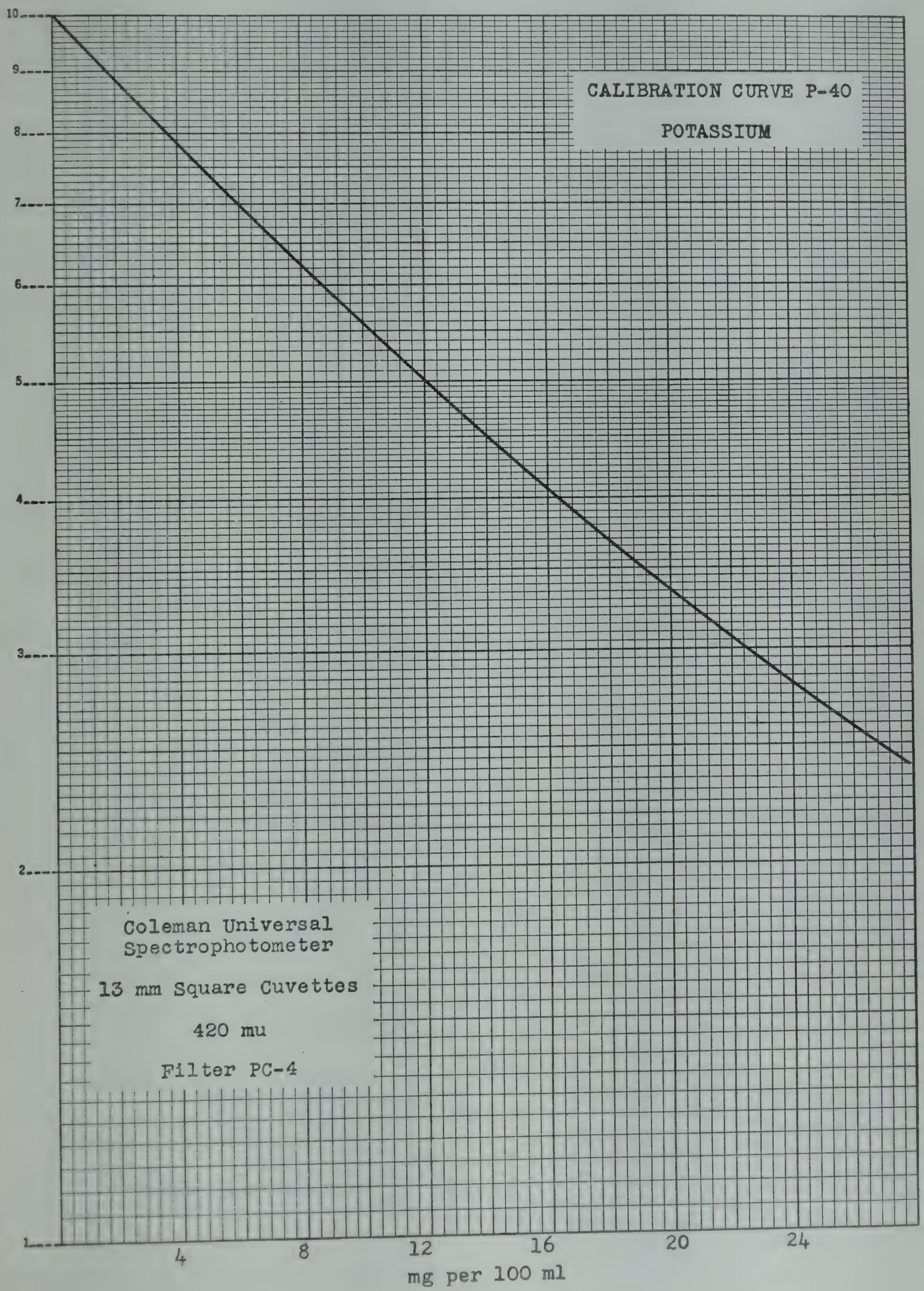
CALIBRATION CURVE P-40
POTASSIUM
Coleman Universal
Spectrophotometer
13 mm Square Cuvettes
420 mu
Filter PC-4
10
9
8
7
6
5
4
3
2
1
4
8
12
16
20
24
mg per 100 ml

Meter Reading	0	1	2	3	4	5	6	7	8	9
0										
10										
20										
30										
40										
50										
60										
70										
80										
90										

POTASSIUM PERMANGANATE
(Volumetric Standardization)

A spectrophotometric adaptation based on the method of Hoffman (1940; 1941)

PRINCIPLE

The transmittancy of a potassium permanganate solution of approximate normality is compared by means of a calibration curve with the transmittancy of permanganate solutions of known strength. The solution may then be used, employing the factor obtained from the calibration table, or may be adjusted to the exact normality by suitable dilution.

TEST PROCEDURE

Special Apparatus

Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.

Reagent

1. **Potassium Permanganate Stock Solutions.** All potassium permanganate solutions should be prepared in the same manner as outlined for the volumetric 0.1 N potassium permanganate (Reagent #4a) in the calibration procedure. For an approximate 0.1 N solution use 3.5 gm per 1000 ml; for a 0.05 N solution use 1.8 gm and for a 0.01 N solution use 0.35 gm. The exact normality factor of these solutions is determined as outlined in the test procedure.

Technic

Note: The temperature of the unknown solution must be exactly the same as that of the standard solutions used when constructing the calibration curve. In the author's laboratory a temperature of 21° C. is always maintained when standardizing volumetric solutions.

1. Dilute the stock permanganate solutions (1) as follows:
   For approximate 0.1 N—dilute 2.5 ml to 500 ml with distilled water
   For approximate 0.05 N—dilute 5 ml to 500 ml with distilled water
   For approximate 0.01 N—dilute 25 ml to 500 ml with distilled water.
2. In a cuvette (sample) place 10 ml of the diluted permanganate solution.
3. Measure the transmittancy of the sample against distilled water set at 100% transmission at a wave length of 530 mμ and obtain the factor from the calibration table.

Note: The permanganate solution (if the factor is greater than 1) may be diluted to the proper normality by the following formula:

$$\frac{1000}{\text{factor}} = \text{Number of ml to be diluted to 1000 ml}$$

It is preferable, however, to use the permanganate without further dilution and to correct results by means of the factor.

CALIBRATION PROCEDURE

Special Apparatus

Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.

Reagents

2. **Sodium Oxalate, Volumetric 0.1 N.** Dry sodium oxalate ($Na_2C_2O_4$—National Bureau of Standards) in a hot air oven at 100–105° C. for 1 hour; cool in desiccator to constant weight; transfer exactly 6.7005 gm to a 1000 ml volumetric flask; add approximately 900 ml distilled water and shake until dissolved; add 5 ml concentrated sulfuric acid (H_2SO_4—Grasselli, A.C.S.); dilute to volume with distilled water and mix thoroughly. Preserve in resistant-glass bottle. Stability indefinite.
3. **Sulfuric Acid, 25% by Volume.** In a 250 ml beaker place 75 ml distilled water and slowly add, with constant stirring, 25 ml concentrated sulfuric acid (H_2SO_4—Grasselli, A.C.S.); allow to cool and adjust to a volume of 100 ml with distilled water. Preserve in glass-stoppered bottle.
4. **Potassium Permanganate Standards**
   (a) Potassium permanganate, volumetric 0.1 N; in a 2000 ml Florence flask with ground glass joint place 3.5 gm crystalline potassium permanganate ($KMnO_4$—Baker & Adamson, A.C.S.); add 1000 ml distilled water and shake until dissolved; connect a reflux condenser and heat to boiling; digest at just below the boiling point for 2 hours; allow to stand over night and filter with gentle suction through a fritted glass funnel; transfer to a Pyrex glass-stoppered bottle and allow to stand for 1 week before standardizing. In a 500 ml titration flask place approximately 200 ml distilled water and heat to 70° C.; add exactly 20 ml 0.1 N sodium oxalate (2) and 15 ml 25% sulfuric acid (3); titrate the hot solution at once with the permanganate to the first permanent pink color (the temperature should never fall below 70° C.—reheat to maintain this temperature if necessary); dilute the permanganate accordingly and re-titrate—25 ml of 0.1 N sodium oxalate require 25 ml of 0.1 N potassium permanganate. Preserve in a brown Pyrex glass-stoppered bottle in the dark. Check normality at monthly intervals.
   (b) Calibration standards. In a series of three accurately calibrated 500 ml volumetric

flasks place respectively 2.0, 2.5 and 3.0 ml of the accurately standardized 0.1 N potassium permanganate (4a); dilute to volume with distilled water and mix thoroughly. These standards represent normalities of N/2000 with factors of 0.8, 1.0 and 1.2. Prepare just before use and proceed immediately as follows.

Technic

1. Transfer a suitable portion of each calibration standard (4b) to a correspondingly marked cuvette.
2. Set distilled water at 100% transmission at a wave length of 530 mμ and record the transmittancy of the calibration standards (4b). The transmittancy of the solution with the factor of 1.2 should be approximately 49%.
3. Plot the observed values on semi-log graph paper using the factors as ordinates so that each division unit represents a factor change of 0.01—the points fall on a straight line (Calibration Curve P-45).
4. Prepare a calibration table from the T-C values obtained from the plotted curve.

PROCEDURE FOR USE WITH LINEAR-SCALE PHOTOELECTRIC COLORIMETERS (SHEARD-SANFORD TYPE)

TEST PROCEDURE

Dilute the specified amounts of the stock permanganate solutions (1) to 200 ml with distilled water (instead of 500 ml); proceed as outlined; set distilled water at 100 and use a green filter.

CALIBRATION PROCEDURE

In preparing the calibration standards dilute 2.0, 2.5 and 3.0 ml of the accurately standardized 0.1 N potassium permanganate (4a) to 200 ml with distilled water (instead of 500 ml); proceed as outlined—use a green filter; plot the observed readings on semi-log graph paper—the points fall on a straight line.

PROCEDURE FOR USE WITH LOGARITHMIC-SCALE PHOTOELECTRIC COLORIMETERS (KLETT-SUMMERSON TYPE)

TEST PROCEDURE

Dilute the specified amounts of the stock permanganate solutions (1) to 200 ml with distilled water (instead of 500 ml); proceed as outlined; set distilled water at 0 and use a green filter.

CALIBRATION PROCEDURE

In preparing the calibration standards dilute 2.0, 2.5 and 3.0 ml of the accurately standardized 0.1 N potassium permanganate (4a) to 200 ml with distilled water (instead of 500 ml); proceed as outlined—use a green filter; plot the observed readings on linear graph paper—the points fall on a straight line.

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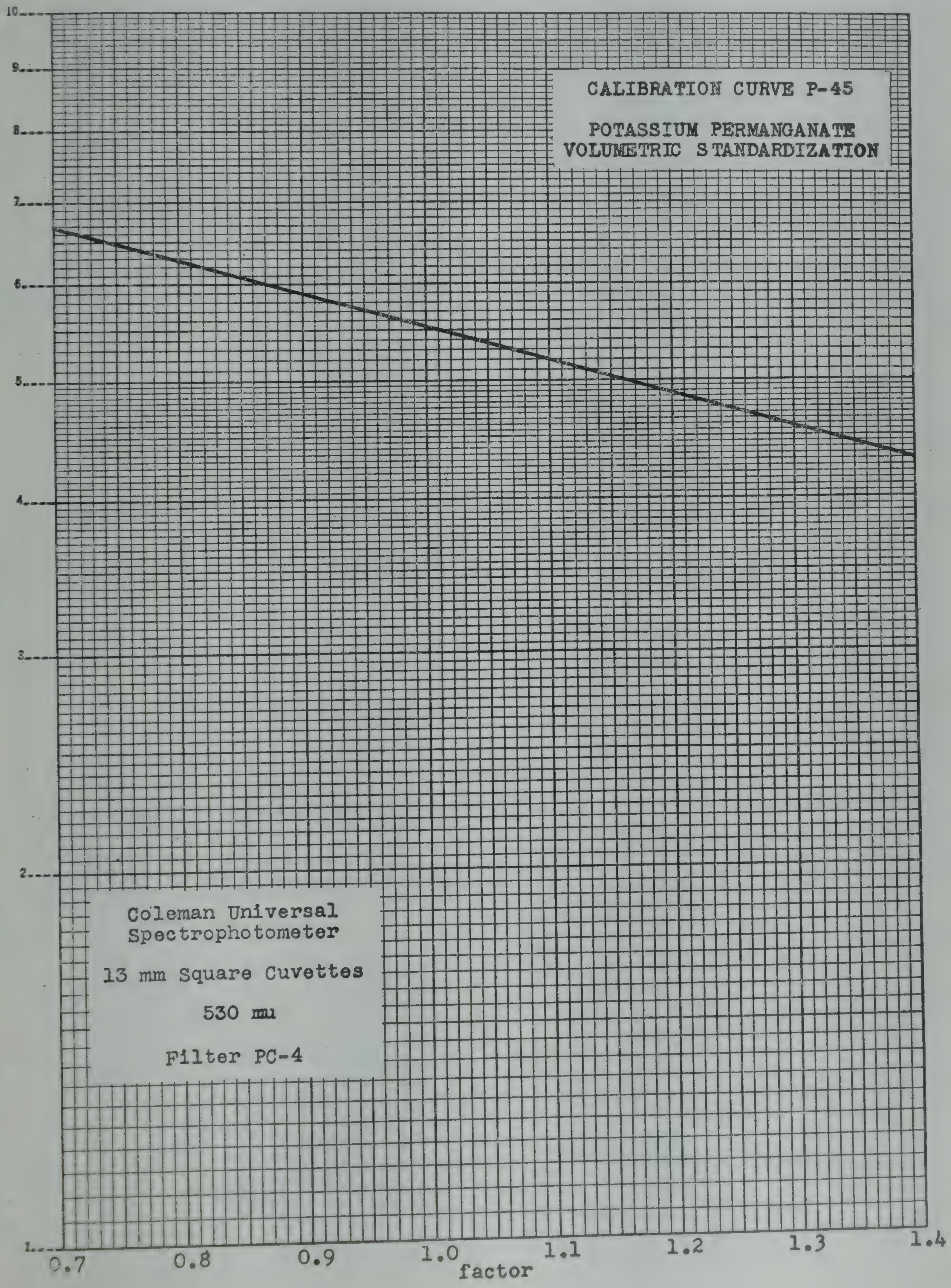
CALIBRATION CURVE P-45
POTASSIUM PERMANGANATE VOLUMETRIC STANDARDIZATION
Coleman Universal Spectrophotometer
13 mm Square Cuvettes
530 mμ
Filter PC-4
10
9
8
7
6
5
4
3
2
1
0.7
0.8
0.9
1.0
1.1
1.2
1.3
1.4
factor

Meter Reading	0	1	2	3	4	5	6	7	8	9
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20										
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PREGNANDIOL, TOTAL

A spectrophotometric adaptation based on the methods and modifications of Venning (1938), Shinowara and Reinhart (1939), Talbot, Berman, MacLachlan and Wolfe (1941), Astwood and Jones (1941), Reinhart, Reel and Shinowara (1941), Davis and Fugo (1947) and Goldzieher (1948)

PRINCIPLE

Urine is subjected to acid hydrolysis to liberate free pregnandiol which is simultaneously extracted by toluene. Interfering impurities and pigments are removed by treatment with alcoholic alkali. Pregnandiol is then taken up in acetone, precipitated by sodium hydroxide, redissolved in alcohol and after evaporation of the solvent, is treated with acetyl chloride and zinc chloride to produce an orange-yellow color.

DETERMINATION IN URINE

SAMPLE

Aliquot of a 24 or 48-hour specimen. Preserve with 0.1 ml tricresol per liter. Keep cold during collection and refrigerate immediately upon completion of the collection period. Reject specimens with any evidence of decomposition. The analysis must be started and carried through the extraction procedure within 12 hours after collection of the sample.

TEST PROCEDURE

Special Apparatus

Boiling flask, 500 ml, Pyrex, flat-bottom with ground glass joint connected to a 400–500 mm Liebig water-cooled reflux condenser.
Separatory funnels, 250 and 500 ml, pear-shaped.
Fritted glass filters, Pyrex, 60 ml, porosity M.
Graduated centrifuge tubes—15 ml; Pyrex, glass-stoppered; the 10 ml graduation must be accurately calibrated.
Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.

Reagents

1. **Toluene** ($C_6H_5CH_3$—Baker & Adamson, A.C.S.).
2. **Hydrochloric Acid, Concentrated** (HCl—Baker & Adamson, A.C.S.).
3. **Sodium Hydroxide, Volumetric 0.1 N.** Prepare by diluting slightly more than the required amount of concentrated carbonate-free sodium hydroxide solution (see Appendix) and standardize against 0.1 N acid using phenolphthalein as indicator. Preserve in ceresin-lined bottle.
4. **Sodium Hydroxide, 2% in Methanol.** Dissolve 5 gm sodium hydroxide pellets (NaOH—J. T. Baker, CP Analyzed, Low-carbonate) in 100 ml absolute methyl alcohol (CH_3OH—Merck, A.C.S.); allow to stand over night and filter through a dry fritted glass filter into a dry flask; titrate against standard 0.5 N acid using phenolphthalein as indicator; adjust accordingly and re-titrate—20 ml of 0.5 N acid require 20 ml of 2% alkali. Preserve in ceresin-lined bottle. Prepare fresh weekly.
5. **Acetone** ($(CH_3)_2CO$—Baker & Adamson, A.C.S.).
6. **Petroleum Ether** (B. R. 30–60°—Baker & Adamson, A.C.S.).
7. **Ethyl Alcohol, Absolute** (C_2H_5OH—U. S. Industrial Chemicals, U.S.P.).
8. **Zinc Chloride Reagent.** Place 38 gm anhydrous fused zinc chloride ($ZnCl_2$—Baker & Adamson, Reagent, Fused Sticks) in a 250 ml Erlenmeyer flask; heat over a free flame until all moisture and fumes are driven off and a clear melt results; rotate the flask so that the melt is evenly distributed over the sides of the lower half of the flask; allow to cool to less than 100° C.; add 50 ml glacial acetic acid (CH_3COOH—Baker & Adamson, A.C.S.) and heat gently until solution is complete; transfer to a 100 ml mixing cylinder; rinse out the flask with small portions of acetic acid and transfer the rinsings to the cylinder; dilute to 100 ml with acetic acid. Preserve in glass-stoppered bottle with adequate precautions against absorption of moisture.
9. **Acetyl Chloride** (CH_3COCl—J. T. Baker, CP Analyzed).

Technic

Note: For concentrations of pregnandiol less than 0.04 mg per 100 ml, use a larger sample with correspondingly increased amounts of reagents in Steps 1–16 inclusive and divide the values obtained from the calibration table by the appropriate factor. It is essential that strict adherence be paid to time and temperature details.

1. In a 500 ml flat-bottom boiling flask with ground glass joint place
   100 ml filtered urine
   50 ml toluene (1)
   10 ml concentrated hydrochloric acid (2)
   2 glass beads.
2. Connect a Liebig water-cooled reflux condenser and boil vigorously over an electric hot plate for 15 minutes.
3. Carefully remove the flask from the hot plate *without shaking* and cool to room temperature under running tap water.
4. Transfer the contents of the flask to a 500 ml separatory funnel and draw off the lower urine layer into a 250 ml separatory funnel.

5. Rinse out the boiling flask with 10 ml hot toluene (1) and decant the washing into the 250 ml separatory funnel; shake with a swirling motion for 1 minute and return the entire contents to the original 500 ml separatory funnel containing the toluene emulsion.
6. Mix and again draw off the urine layer into the same 250 ml separatory funnel, add 10 ml hot toluene (1), shake as before for 1 minute and return the contents to the 500 ml separatory funnel.
7. Draw off the urine layer and discard.
8. Wash the combined toluene emulsion phase in the 500 ml funnel twice with 15 ml portions 0.1 N sodium hydroxide (3)—discard the washings.
9. Wash twice more with 15 ml portions distilled water—discard the washings.
10. Transfer the toluene emulsion to a 125 ml Erlenmeyer flask containing two glass beads, rinsing the funnel with a small portion of toluene (1) and adding the washing to the flask—occasionally a troublesome emulsion that does not separate may occur at this point; break up by adding 1–2 drops aerosol.
11. Boil over an electric hot plate until the layer of emulsion has disappeared and the toluene mixture is boiling smoothly—carry out under a fume hood with adequate ventilation.
12. Slowly add 10 ml 2% sodium hydroxide in methanol (4).
13. Evaporate over an electric hot plate until a granular precipitate appears and the volume is approximately half that of the original volume of toluene—the solution should have a yellow or greenish color.
14. Filter, while hot, through a fritted glass filter with gentle suction—if the filtrate has an orange, pink or brown tinge, repeat Steps 12–14 inclusive, filtering through the same funnel into the same flask—this procedure must be repeated until the filtrate is yellow or yellow green.
15. Without removing the filter from the receiving flask, rinse out the Erlenmeyer flask with 15 ml hot toluene (1) and pour the washing over the precipitate in the filter—the combined volume of filtrate and washing is now in one flask.
16. Evaporate to dryness over an electric hot plate; use a gentle air stream to drive off the last traces of toluene and avoid charring the residue—carry out under a fume hood with adequate ventilation.
17. Add 5 ml acetone (5) to the residue and warm gently over the hot plate until solution is complete.
18. While the flask is still on the hot plate, slowly add 20 ml 0.1 N sodium hydroxide (3) and boil for 3 minutes.
19. Place the flask in the refrigerator at 5° C. for 60 minutes—longer does no harm.
20. Remove the flask from the refrigerator and filter the contents through a fritted glass filter with gentle suction; rinse out the flask with 15 ml distilled water and pour the washing over the precipitate in the filter.
21. Wash the precipitate with 10 ml petroleum ether (6).
22. Replace the receiving flask with another dry flask and add 10 ml hot absolute ethyl alcohol (7) to the filter; if the precipitate shows any discoloration, re-precipitate by adding 40 ml distilled water, heat to boiling, allow to cool, re-filter and re-dissolve in 10 ml hot absolute ethyl alcohol—repeat this procedure until the precipitate is white.
23. Transfer the alcohol filtrate to a 15 ml graduated Pyrex glass-stoppered centrifuge tube (sample), washing out the flask with several small portions hot alcohol, adding the washings to the centrifuge tube.
24. Place the centrifuge tube in a glycerine bath and allow to remain until all the alcohol has evaporated; use a gentle air stream to drive off the last traces of alcohol—carry out under a fume hood with adequate ventilation.
25. Remove the tube from the bath and allow to cool to room temperature.
26. Add 6 ml zinc chloride reagent (8)
    2.5 ml acetyl chloride (9) from a burette.
27. In another 15 ml graduated Pyrex glass-stoppered centrifuge tube (reagent blank) place
    6 ml zinc chloride reagent (8)
    2.5 ml acetyl chloride (9) from a burette.
28. Stopper the tubes tightly and mix thoroughly by inversion.
29. Place the tubes in a water bath at 50° C.—the level of the water in the bath should not be above the shoulders of the tubes.
30. Allow the tubes to remain in the water bath for 30 minutes—an orange-yellow color develops in the sample tube.
31. Without removing the stoppers, place the tubes in cold water and allow to remain for 20 minutes.
32. Wipe all the water off the outside of the tubes.
33. Adjust the volume in each tube to exactly 10 ml with zinc chloride reagent (8), stopper and mix thoroughly by inversion.
34. Transfer the contents of the tubes to correspondingly marked cuvettes.
35. Measure the transmittancy of the sample against the reagent blank set at 100% transmission at a wave length of **400** mμ and obtain the concentration of pregnandiol from the calibration table.

Calculation

$$\frac{\text{Volume of 24-hour specimen}}{100} \times \text{Pregnandiol in mg per 100 ml} = \text{Mg pregnandiol excreted in 24 hours}$$

CALIBRATION PROCEDURE

Special Apparatus

Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.
Graduated centrifuge tubes—15 ml; Pyrex, glass-stoppered; the 10 ml graduation must be accurately calibrated.
Micro burette, 1 ml in 0.001 ml.

Reagents

7. **Ethyl Alcohol, Absolute.** Same as for Test Procedure.
8. **Zinc Chloride Reagent.** Same as for Test Procedure.
9. **Acetyl Chloride.** Same as for Test Procedure.
10. **Pregnandiol Standards**
    (a) Pregnandiol stock standard (1 ml contains 0.5 mg pregnandiol). In a 100 ml volumetric flask place 50 mg pregnandiol ($C_{21}H_{36}O_2$); add approximately 80 ml absolute ethyl alcohol (7); shake until dissolved and dilute to volume with same. Preserve in refrigerator. Stability indefinite.
    (b) Calibration standards. In a series of five accurately calibrated 15 ml graduated glass-stoppered centrifuge tubes place respectively 0.2, 0.4, 0.6, 0.8 and 1 ml of the stock standard (10a) measured from a micro burette. These standards represent concentrations of pregnandiol equivalent to 0.1, 0.2, 0.3, 0.4, and 0.5 mg per 100 ml sample. Prepare just before use and proceed immediately as follows.

Technic

1. Place the centrifuge tubes containing the calibration standards (10b) in a glycerine bath and allow to remain until all the alcohol has evaporated; use a gentle air stream to drive off the last traces of alcohol.
2. Remove the tubes from the bath and allow to cool to room temperature.
3. To each tube add
    6 ml zinc chloride reagent (8)
    2.5 ml acetyl chloride (9) from a burette.
4. Proceed exactly as outlined in the Test Procedure, Steps 27–34 inclusive.
5. Set the zero concentration standard (reagent blank) at 100% transmission at a wave length of 400 mμ and record the transmittancy of the 0.1, 0.2, 0.3, 0.4 and 0.5 mg per cent standards. The transmittancy of the 0.5 mg standard should be approximately 19%.
6. Plot the observed values on semi-log graph paper —the points fall on a smooth curve which passes through the origin (Calibration Curve P-47).
7. Prepare a calibration table from the T-C values obtained from the plotted curve.

PROCEDURE FOR USE WITH LINEAR-SCALE PHOTOELECTRIC COLORIMETERS (SHEARD-SANFORD TYPE)

TEST PROCEDURE

Exactly as outlined; set the reagent blank at 100 and use a blue filter.

CALIBRATION PROCEDURE

Exactly as outlined—use a blue filter; plot the observed readings on semi-log graph paper—the points fall on a smooth curve passing through the origin.

PROCEDURE FOR USE WITH LOGARITHMIC-SCALE PHOTOELECTRIC COLORIMETERS (KLETT-SUMMERSON TYPE)

TEST PROCEDURE

Exactly as outlined; set the reagent blank at 0 and use a blue filter.

CALIBRATION PROCEDURE

Exactly as outlined—use a blue filter; plot the observed readings on linear graph paper—the points fall on a smooth curve passing through the origin.

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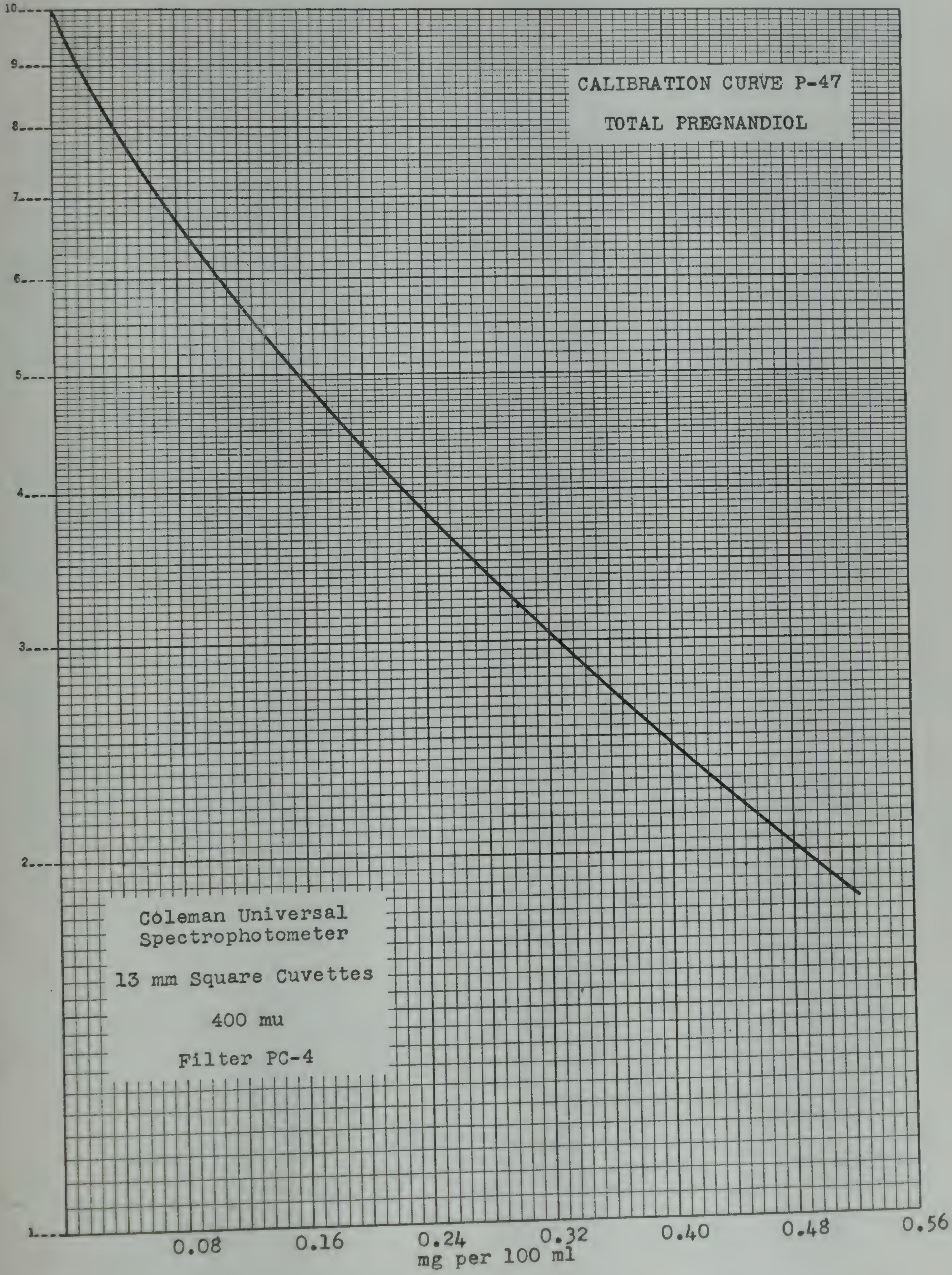
CALIBRATION CURVE P-47
TOTAL PREGNANDIOL
Coleman Universal Spectrophotometer
13 mm Square Cuvettes
400 mu
Filter PC-4
10
9
8
7
6
5
4
3
2
1
0.08
0.16
0.24
0.32
0.40
0.48
0.56
mg per 100 ml

Meter Reading	0	1	2	3	4	5	6	7	8	9
0										
10										
20										
30										
40										
50										
60										
70										
80										
90										

PREGNANDIOL, TOTAL

A spectrophotometric adaptation based on the methods and modifications of Venning (1938), Shinowara and Reinhart (1939), Talbot, Berman, MacLachlan and Wolfe (1941), Astwood and Jones (1941), Reinhart, Reel and Shinowara (1941), Guterman (1944; 1945), Davis and Fugo (1947) and Guterman and Schroeder (1948)

PRINCIPLE

Urine is subjected to acid hydrolysis to liberate free pregnandiol which is simultaneously extracted by toluene. Interfering impurities and pigments are removed by treatment with alcoholic alkali. Pregnandiol is then taken up in acetone, precipitated by sodium hydroxide, redissolved in alcohol and after evaporation of the solvent, taken up in sulfuric acid to produce a pale yellow color (Guterman reaction).

DETERMINATION IN URINE

SAMPLE

Aliquot of a 24 or 48-hour specimen. Preserve with 0.1 ml tricresol per liter. Keep cold during collection and refrigerate immediately upon completion of the collection period. Reject specimens with any evidence of decomposition. The analysis must be started and carried through the extraction procedure within 12 hours after collection of the sample.

TEST PROCEDURE

Special Apparatus

Boiling flask, 500 ml, Pyrex, flat-bottom with ground glass joint connected to a 400–500 mm Liebig water-cooled reflux condenser.
Separatory funnels, 250 and 500 ml, pear-shaped.
Fritted glass filters, Pyrex, 60 ml, porosity M.
Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.

Reagents

1. **Toluene** ($C_6H_5CH_3$—Baker & Adamson, A.C.S.).
2. **Hydrochloric Acid, Concentrated** (HCl—Baker & Adamson, A.C.S.).
3. **Sodium Hydroxide, Volumetric 0.1 N.** Prepare by diluting slightly more than the required amount of concentrated carbonate-free sodium hydroxide solution (see Appendix) and standardize against 0.1 N acid using phenolphthalein as indicator. Preserve in ceresin-lined bottle.
4. **Sodium Hydroxide, 2% in Methanol.** Dissolve 5 gm sodium hydroxide pellets (NaOH—J. T. Baker, CP Analyzed, Low-carbonate) in 100 ml absolute methyl alcohol (CH_3OH—Merck, A.C.S.); allow to stand over night and filter through a dry fritted glass filter into a dry flask; titrate against standard 0.5 N acid using phenolphthalein as indicator; adjust accordingly and re-titrate—20 ml of 0.5 N acid require 20 ml of 2% alkali. Preserve in ceresin-lined bottle. Prepare fresh weekly.
5. **Acetone** ($(CH_3)_2CO$—Baker & Adamson, A.C.S.).
6. **Petroleum Ether** (B. R. 30–60°—Baker & Adamson, A.C.S.).
7. **Ethyl Alcohol, Absolute** (C_2H_5OH—U.S. Industrial Chemicals, U.S.P.).
8. **Sulfuric Acid, Concentrated** (H_2SO_4—Grasselli, A.C.S.).

Technic

Note: For concentrations of pregnandiol less than 0.02 mg per 100 ml, use a larger sample with correspondingly increased amounts of reagents in Steps 1–16 inclusive and divide the values obtained from the calibration table by the appropriate factor.

1. In a 500 ml flat-bottom boiling flask with ground glass joint place
   100 ml filtered urine
   50 ml toluene (1)
   10 ml concentrated hydrochloric acid (2)
   2 glass beads.
2. Connect a Liebig water-cooled reflux condenser and boil vigorously over an electric hot plate for 15 minutes.
3. Carefully remove the flask from the hot plate *without shaking* and cool to room temperature under running tap water.
4. Transfer the contents of the flask to a 500 ml separatory funnel and draw off the lower urine layer into a 250 ml separatory funnel.
5. Rinse out the boiling flask with 10 ml hot toluene (1) and decant the washing into the 250 ml separatory funnel; shake with a swirling motion for 1 minute and return the entire contents to the original 500 ml separatory funnel containing the toluene emulsion.
6. Mix and again draw off the urine layer into the same 250 ml separatory funnel, add 10 ml hot toluene (1), shake as before for 1 minute and return the contents to the 500 ml separatory funnel.
7. Draw off the urine layer and discard.
8. Wash the combined toluene emulsion phase in the 500 ml funnel twice with 15 ml portions 0.1 N sodium hydroxide (3)—discard the washings.
9. Wash twice more with 15 ml portions distilled water—discard the washings.
10. Transfer the toluene emulsion to a 125 ml Erlenmeyer flask containing two glass beads, rinsing the

funnel with a small portion of toluene (1) and adding the washing to the flask—occasionally a troublesome emulsion that does not separate may occur at this point; break up by adding 1–2 drops aerosol.

11. Boil over an electric hot plate until the layer of emulsion has disappeared and the toluene mixture is boiling smoothly—carry out under a fume hood with adequate ventilation.
12. Slowly add 10 ml 2% sodium hydroxide in methanol (4).
13. Evaporate over an electric hot plate until a granular precipitate appears and the volume is approximately half that of the original volume of toluene—the solution should have a yellow or greenish color.
14. Filter, while hot, through a fritted glass filter with gentle suction—if the filtrate has an orange, pink or brown tinge, repeat Steps 12–14 inclusive, filtering through the same funnel into the same flask—this procedure must be repeated until the filtrate is yellow or yellow green.
15. Without removing the filter from the receiving flask, rinse out the Erlenmeyer flask with 15 ml hot toluene (1) and pour the washing over the precipitate in the filter—the combined volume of filtrate and washing is now in one flask.
16. Evaporate to dryness over an electric hot plate, use a gentle air stream to drive off the last traces of toluene and avoid charring the residue—carry out under a fume hood with adequate ventilation.
17. Add 5 ml acetone (5) to the residue and warm gently over the hot plate until solution is complete.
18. While the flask is still on the hot plate, slowly add 20 ml 0.1 N sodium hydroxide (3) and boil for 3 minutes.
19. Place the flask in the refrigerator at 5° C. for 60 minutes—longer does no harm.
20. Remove the flask from the refrigerator and filter the contents through a fritted glass filter with gentle suction; rinse out the flask with 15 ml distilled water and pour the washing over the precipitate in the filter.
21. Wash the precipitate with 10 ml petroleum ether (6).
22. Replace the receiving flask with another dry flask and add 10 ml hot absolute ethyl alcohol (7) to the filter; if the precipitate shows any discoloration, re-precipitate by adding 40 ml distilled water, heat to boiling, allow to cool, re-filter and redissolve in 10 ml hot absolute ethyl alcohol—repeat this procedure until the precipitate is white.
23. Evaporate the alcohol filtrate to dryness over an electric hot plate; use a gentle air stream to drive off the last traces of alcohol—carry out under a fume hood with adequate ventilation.
24. Allow to cool to room temperature; add 10 ml concentrated sulfuric acid (8) to the residue and stir with a thin glass rod.
25. Allow to stand at room temperature for 60 minutes, shaking occasionally.
26. Dilute an aliquot (containing less than 0.1 mg) to 5 ml with concentrated sulfuric acid (8) and transfer to a cuvette (sample)—a 1:10 dilution is usually sufficient.
27. Measure the transmittancy of the sample against concentrated sulfuric acid (8) set at 100% transmission at a wave length of 430 mμ and obtain the value for pregnandiol from the calibration table.

Calculation

Value for pregnandiol from calibration table × dilution factor (from Step 26) = Pregnandiol in mg per 100 ml urine

$$\frac{\text{Volume of 24-hour specimen}}{100} \times \text{Pregnandiol in mg per 100 ml} = \text{Mg pregnandiol excreted in 24 hours}$$

CALIBRATION PROCEDURE

Special Apparatus

Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.

Micro burette, 1 ml in 0.001 ml.

Reagents

7. **Ethyl Alcohol, Absolute.** Same as for Test Procedure.
8. **Sulfuric Acid, Concentrated.** Same as for Test Procedure.
9. **Pregnandiol Standards**
   (a) Pregnandiol stock standard (1 ml contains 0.1 mg pregnandiol). In a 100 ml volumetric flask place 10 mg pregnandiol ($C_{21}H_{36}O_2$); add approximately 80 ml absolute ethyl alcohol (7); shake until dissolved and dilute to volume with same. Preserve in refrigerator. Stability indefinite.
   (b) Calibration standards. In a series of five cuvettes place respectively 0.2, 0.4, 0.6, 0.8, and 1 ml of the stock standard (9a) measured from a micro burette. These standards represent concentrations of pregnandiol equivalent to 0.02, 0.04, 0.06, 0.08 and 0.1 mg per 100 ml sample. Prepare just before use and proceed immediately as follows.

Technic

1. Place the cuvettes containing the calibration standards (9b) in a glycerine bath and allow to remain until all the alcohol has evaporated; use a gentle air stream to drive off the last traces of alcohol.
2. Remove the cuvettes from the bath and allow to cool to room temperature.

3. To each cuvette add 5 ml concentrated sulfuric acid (8) and stir with thin glass rods.
4. Allow to stand at room temperature for 60 minutes, shaking occasionally.
5. Set the zero concentration standard (concentrated sulfuric acid) at 100% transmission at a wave length of **430** mμ and record the transmittancy of the 0.02, 0.04, 0.06, 0.08 and 0.1 mg per cent standards. The transmittancy of the 0.1 mg standard should be approximately 40%.
6. Plot the observed values on semi-log graph paper —the points fall on a straight line which passes through the origin (Calibration Curve P-48).
7. Prepare a calibration table from the T-C values obtained from the plotted curve.

PROCEDURE FOR USE WITH LINEAR-SCALE PHOTOELECTRIC COLORIMETERS (SHEARD-SANFORD TYPE)

TEST PROCEDURE

Exactly as outlined; set concentrated sulfuric acid at 100 and use a blue filter.

CALIBRATION PROCEDURE

Exactly as outlined—use a blue filter; plot the observed readings on semi-log graph paper—the points fall on a straight line passing through the origin.

PROCEDURE FOR USE WITH LOGARITHMIC-SCALE PHOTOELECTRIC COLORIMETERS (KLETT-SUMMERSON TYPE)

TEST PROCEDURE

Exactly as outlined; set concentrated sulfuric acid at 0 and use a blue filter.

CALIBRATION PROCEDURE

Exactly as outlined—use a blue filter; plot the observed readings on linear graph paper—the points fall on a straight line passing through the origin.

Note: For instruments with cells, cuvettes or tubes requiring a final volume of more than 5 ml, double the aliquot in Step 26 and dilute to 10 ml with concentrated sulfuric acid in the test procedure. In the calibration procedure double the concentration of the calibration standards and add 10 ml concentrated sulfuric acid in Step 3.

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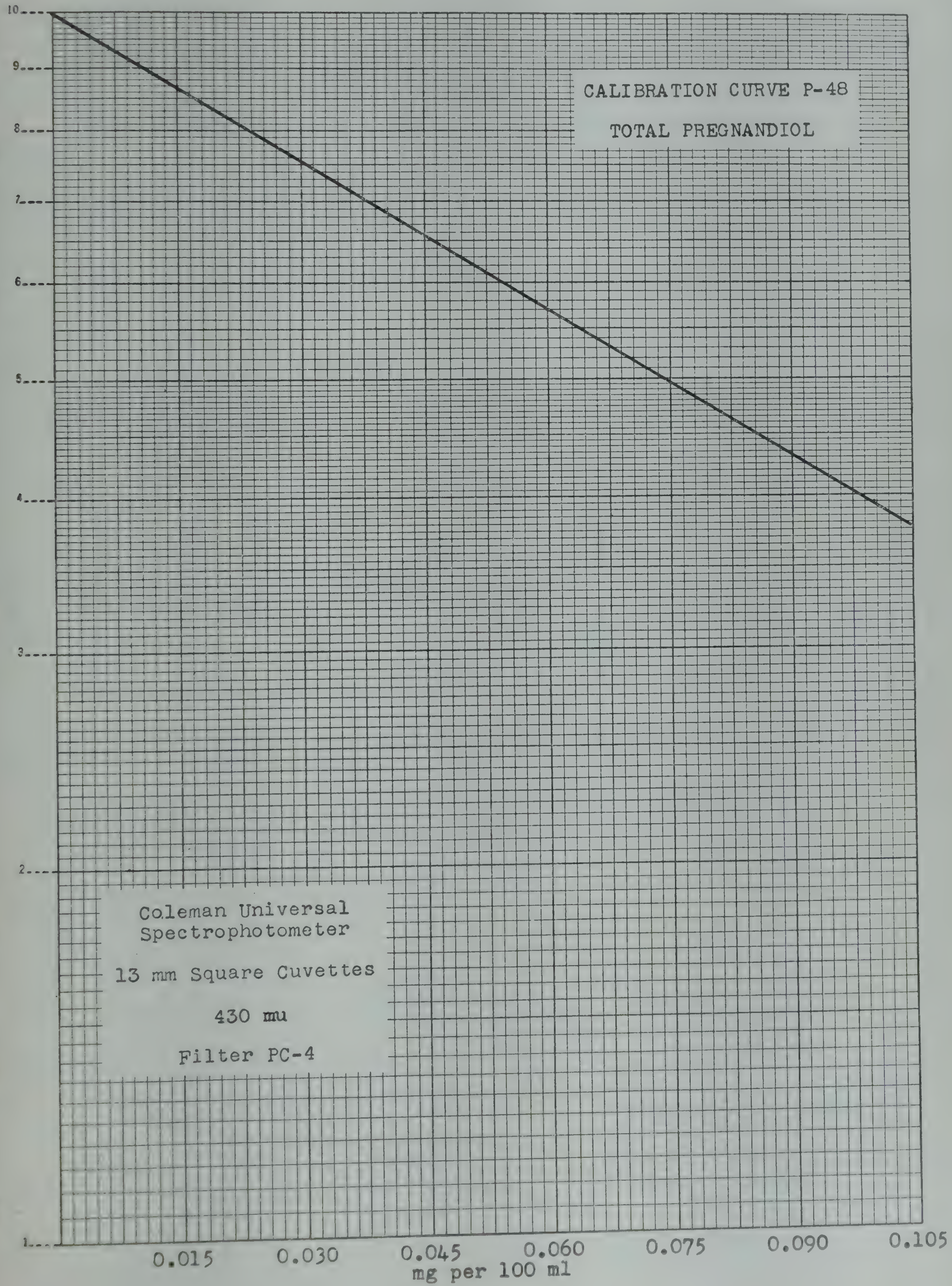
NOTES


CALIBRATION CURVE P-48
TOTAL PREGNANDIOL
Coleman Universal Spectrophotometer
13 mm Square Cuvettes
430 mu
Filter PC-4
10
9
8
7
6
5
4
3
2
1
0.015
0.030
0.045
0.060
0.075
0.090
0.105
mg per 100 ml

Meter Reading	0	1	2	3	4	5	6	7	8	9
0										
10										
20										
30										
40										
50										
60										
70										
80										
90										

PROTEIN, TOTAL

A spectrophotometric adaptation based on the methods and modifications of Kingsley (1939; 1942), Robinson and Hogden (1940), Weichselbaum (1946), Gornall, Bardawill and David (1949)

PRINCIPLE

Protein in plasma or serum is treated directly with copper sulfate in the presence of alkaline sodium potassium tartrate to form a reddish-purple complex (biuret reaction).

DETERMINATION IN PLASMA OR SERUM

SAMPLE

Plasma or serum from blood withdrawn during postabsorptive state. Anticoagulant—potassium oxalate. There must be no trace of hemolysis. Plasma or serum must be separated from the red blood cells as soon as possible.

TEST PROCEDURE

Special Apparatus

Ostwald-Folin pipettes accurately calibrated "to contain" 0.5 ml.

Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.

Reagents

1. **Sodium Chloride, 0.85% Aqueous.** Dissolve 8.5 gm sodium chloride (NaCl—Merck, Special for Biological Work) in distilled water and dilute to 1000 ml.
2. **Biuret Reagent (Gornall-Bardawill-David).** In a 1000 ml volumetric flask place 1.5 gm crystalline copper sulfate ($CuSO_4 \cdot 5H_2O$—Baker & Adamson, A.C.S., Fine Crystals) and 6 gm sodium potassium tartrate ($NaKC_4H_4O_6 \cdot 4H_2O$—Baker & Adamson, A.C.S.); add approximately 500 ml distilled water and shake until dissolved; add, with constant agitation of the flask, 300 ml 2.5 N sodium hydroxide (a) and mix; add 1 gm potassium iodide (KI—Mallinckrodt, A.C.S.) and shake until dissolved; dilute to volume with distilled water. Preserve in ceresin-lined bottle. Stability of a properly prepared reagent is indefinite—discard if a black or reddish precipitate forms.
   - (a) Sodium hydroxide, volumetric 2.5 N; prepare by diluting slightly more than the required amount of concentrated carbonate-free sodium hydroxide solution (see Appendix); dilute 20 ml of this approximately 2.5 N solution to 100 ml; titrate against standard 0.5 N acid using phenolphthalein as indicator; adjust accordingly and re-titrate—20 ml of 0.5 N acid require 20 ml of a 1:5 dilution of 2.5 N alkali. Preserve in ceresin-lined bottle.

Technic

Note: For concentrations of total protein in excess of 10%, use 1 ml diluted sample and 1 ml sodium chloride in Step 2 and multiply the values obtained from the calibration table by 2.

1. In a test tube place
   9.5 ml 0.85% sodium chloride (1)
   0.5 ml sample from an Ostwald-Folin pipette, thoroughly rinsing out the pipette by drawing in and expelling some of the mixture several times and mix.
2. In an 18 × 150 mm Pyrex test tube (sample) place
   2 ml diluted sample.
3. In another 18 × 150 mm Pyrex test tube (reagent blank) place
   2 ml 0.85% sodium chloride (1).
4. To each tube add 8 ml biuret reagent (2) and mix by whirling.
5. Place tubes in water bath at 25° C. for 30 minutes.

Note: An occasional highly lipemic sample may show a turbidity at this point; add an equal volume of petroleum ether to the colored solution and extract the lipids by gently inverting the tube until the aqueous phase is clear; centrifuge at 1500 rpm for 3 minutes, transfer the aqueous layer to a cuvette and proceed with Step 6.

6. Measure the transmittancy of the sample against the reagent blank set at 100% transmission at a wave length of 540 mμ and obtain the concentration of total protein from the calibration table.

CALIBRATION PROCEDURE

Special Apparatus

Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.

Reagents

1. **Sodium Chloride, 0.85% Aqueous.** Same as for Test Procedure.
2. **Biuret Reagent (Gornall-Bardawill-David).** Same as for Test Procedure.
3. **Total Protein Standards**
   - (a) Protein stock standard. Determine the total protein in a well mixed sample of pooled human sera by the Kjeldahl procedure (Method P-51).
   - (b) Calibration standards. In a series of four accurately calibrated 100 ml volumetric flasks place respectively volumes of pooled sera (3a) containing 120, 240, 360 and 480 mg protein; dilute to volume with 0.85% sodium chloride (1). These standards rep-

resent concentrations of protein equivalent to 2.4, 4.8, 7.2 and 9.6%. Prepare just before use.

Technic

1. Transfer 2 ml of each calibration standard (3b) to a correspondingly marked 18 × 150 mm Pyrex test tube.
2. Proceed exactly as outlined in the Test Procedure, Steps 3–5 inclusive.
3. Remove tubes from water bath and immediately set the zero concentration standard at 100% transmission at a wave length of 540 mμ and record the transmittancy of the 2.4, 4.8, 7.2 and 9.6% standards. The transmittancy of the 9.6% standard should be approximately 45.5%.
4. Plot the observed values on semi-log graph paper—the points fall on a straight line which passes through the origin (Calibration Curve P-50A).
5. Prepare a calibration table from the T-C values obtained from the plotted curve.

Method P-50b

DETERMINATION IN CEREBROSPINAL FLUID

SAMPLE

The sample should be clear and colorless—the presence of blood or heavy bacterial growth invalidates the results. The analysis should not be unduly delayed.

TEST PROCEDURE

Special Apparatus

Ostwald-Folin pipettes accurately calibrated "to deliver" 2 ml.

Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.

Reagent

2. **Biuret Reagent (Gornall-Bardawill-David).** Same as for blood.

Technic

Note: For concentrations of total protein in excess of 500 mg per 100 ml, use 1 ml cerebrospinal fluid and 1 ml distilled water in Step 1 and multiply the values from the calibration table for cerebrospinal fluid by 2 to obtain the concentration of total protein in mg per 100 ml.

1. In an 18 × 150 mm Pyrex test tube (sample) place
   2 ml clear cerebrospinal fluid
   8 ml biuret reagent (2) and mix by whirling.
2. In another 18 × 150 mm Pyrex test tube (reagent blank) place
   2 ml distilled water
   8 ml biuret reagent (2) and mix by whirling.
3. Place tubes in water bath at 25° C. for 30 minutes.
4. Measure the transmittancy of the sample against the reagent blank set at 100% transmission at a wave length of 540 mμ and obtain the concentration of total protein from the calibration table for cerebrospinal fluid.

CALIBRATION PROCEDURE

This calibration procedure need not be carried out. A calibration curve (Calibration Curve P-50B) may be constructed by substituting the values 120, 240, 360 and 480 mg per 100 ml for the transmittancy readings recorded for the 2.4, 4.8, 7.2 and 9.6% concentrations in the calibration curve for blood.

PROCEDURE FOR USE WITH LINEAR-SCALE PHOTOELECTRIC COLORIMETERS (SHEARD-SANFORD TYPE)

TEST PROCEDURE

Exactly as outlined; set the reagent blank at 100 and use a green filter.

CALIBRATION PROCEDURE

Exactly as outlined—use a green filter; plot the observed readings on semi-log graph paper—the points fall on a straight line passing through the origin.

PROCEDURE FOR USE WITH LOGARITHMIC-SCALE PHOTOELECTRIC COLORIMETERS (KLETT-SUMMERSON TYPE)

TEST PROCEDURE

Exactly as outlined; set the reagent blank at 0 and use a green filter.

CALIBRATION PROCEDURE

Exactly as outlined—use a green filter; plot the observed readings on linear graph paper—the points fall on a straight line passing through the origin.

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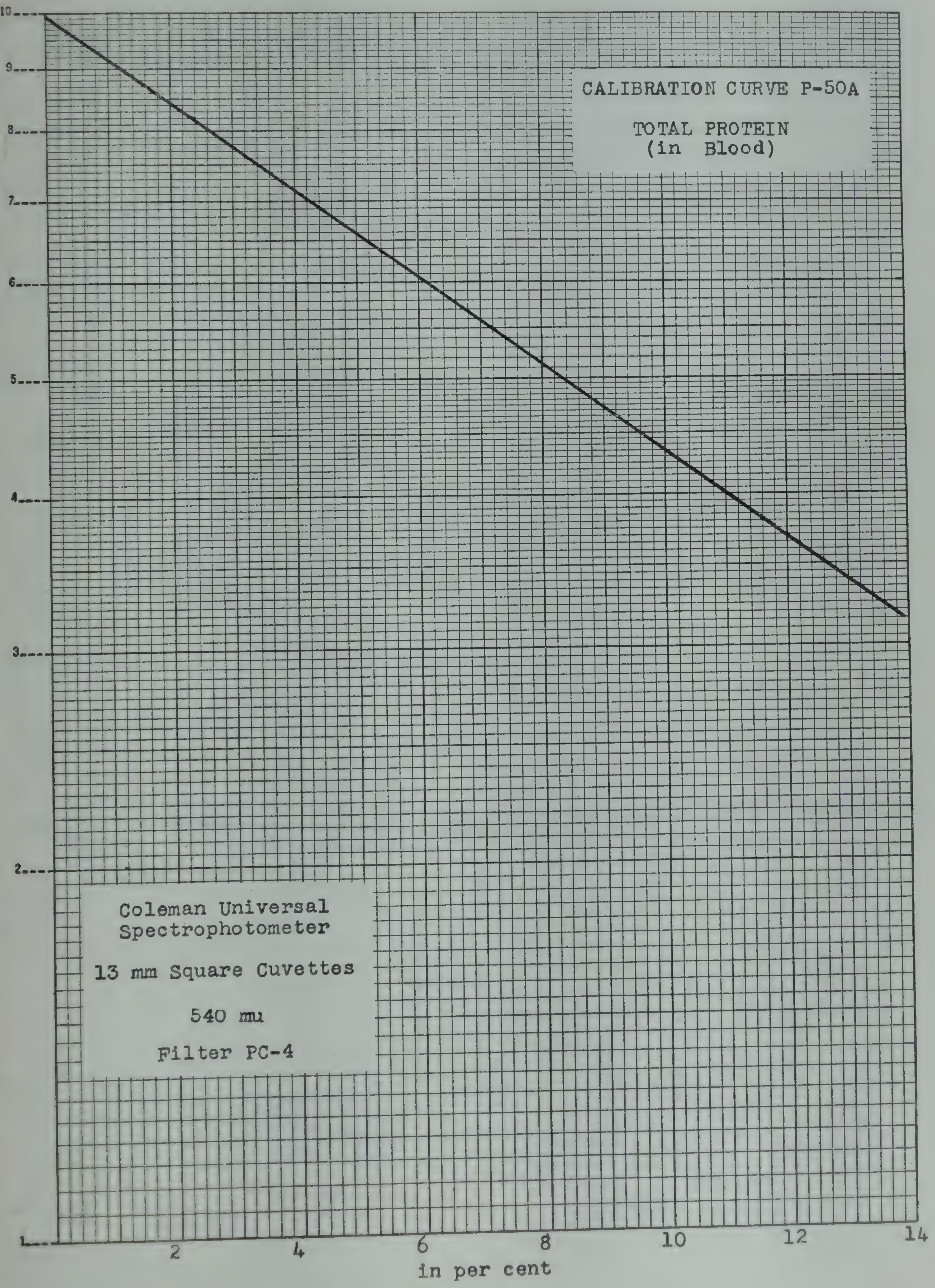
CALIBRATION CURVE P-50A
TOTAL PROTEIN
(in Blood)
Coleman Universal
Spectrophotometer
13 mm Square Cuvettes
540 mu
Filter PC-4
10
9
8
7
6
5
4
3
2
1
2
4
6
8
10
12
14
in per cent

Meter Reading	0	1	2	3	4	5	6	7	8	9
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10										
20										
30										
40										
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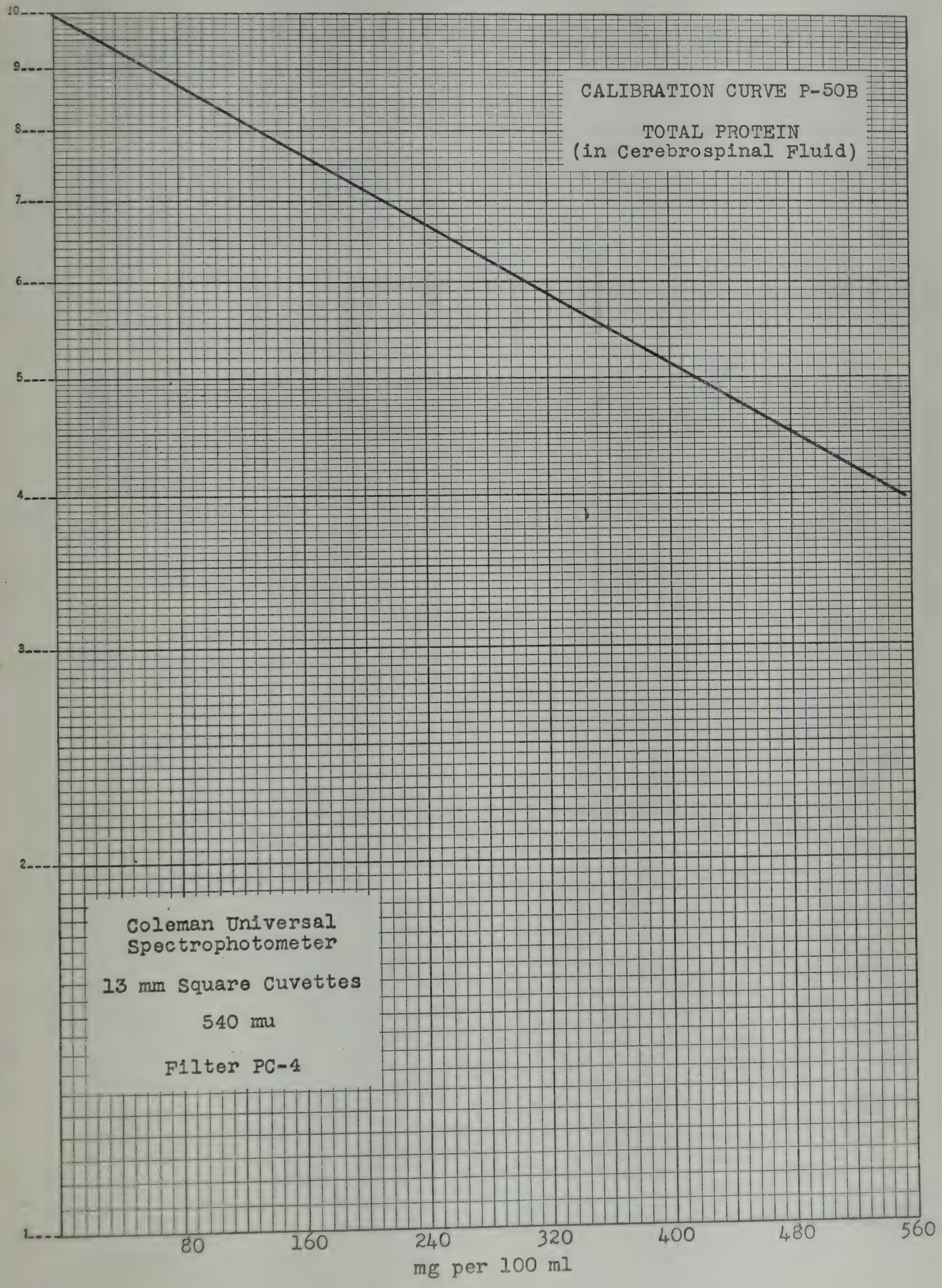
70										
80										
90										


CALIBRATION CURVE P-50B
TOTAL PROTEIN
(in Cerebrospinal Fluid)
Coleman Universal Spectrophotometer
13 mm Square Cuvettes
540 mu
Filter PC-4
10
9
8
7
6
5
4
3
2
1
80
160
240
320
400
480
560
mg per 100 ml

Meter Reading	0	1	2	3	4	5	6	7	8	9
0										
10										
20										
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60										
70										
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PROTEIN, TOTAL

Total protein may be determined in plasma or serum by the micro-Kjeldahl technic as follows:

1. Determine total nitrogen in plasma or serum as outlined in Method N-16b, Calibration Curve N-16B.
2. Determine nonprotein nitrogen in plasma or serum as outlined in Method N-10.
3. Calculation—

Total nitrogen in mg per 100 ml − Nonprotein nitrogen in mg per 100 ml = Total protein nitrogen in mg per 100 ml

Total protein nitrogen in mg per 100 ml × 0.00625 = Total protein in per cent

NOTES

NOTES

PROTEIN, TOTAL

A spectrophotometric adaptation based on the methods and modifications of Wu (1922), Wu and Ling (1927), Folin and Ciocalteu (1927), Greenberg (1929), Andersch and Gibson (1933) and Greenberg and Mirolubova (1936)

PRINCIPLE

Protein in plasma or serum is heated with alkali and treated directly with phosphomolybdotungstic acid phenol reagent which reacts with the phenol group of the tyrosine in each protein to produce an intense blue color. One milligram tyrosine produces a color equivalent to the color produced by 11.5 mg total protein. This method should be used only when the amino acid concentration of the blood is not increased; in such cases either the biuret method (Method P-50) or the micro Kjeldahl method (Method P-51) should be used.

DETERMINATION IN PLASMA OR SERUM

SAMPLE

Plasma or serum from blood withdrawn during postabsorptive state. Anticoagulant—potassium oxalate. There must be no trace of hemolysis. Plasma or serum must be separated from the red blood cells as soon as possible.

TEST PROCEDURE

Special Apparatus

Graduated test tubes—10 ml in 0.1 ml; the 10 ml graduation must be accurately calibrated.

Ostwald-Folin pipettes accurately calibrated "to contain" 0.5 ml.

Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.

Reagents

1. **Sodium Chloride, 0.85% Aqueous.** Dissolve 8.5 gm sodium chloride (NaCl—Merck, Special for Biological Work) in distilled water and dilute to 1000 ml.
2. **Sodium Hydroxide, Volumetric 2.5 N.** Prepare by diluting slightly more than the required amount of concentrated carbonate-free sodium hydroxide solution (see Appendix); dilute 20 ml of this approximately 2.5 N solution to 100 ml; titrate against standard 0.5 N acid using phenolphthalein as indicator; adjust accordingly and retitrate—20 ml of 0.5 N acid require 20 ml of a 1:5 dilution of 2.5 N alkali. Preserve in ceresin-lined bottle.
3. **Phenol Reagent (Folin-Ciocalteu).** In a 1500 ml boiling flask with ground joint place 500 ml distilled water; add 100 gm sodium tungstate ($Na_2WO_4 \cdot 2H_2O$—Mallinckrodt, Folin Special) and shake until dissolved; add 25 gm sodium molybdate ($Na_2MoO_4 \cdot 2H_2O$—Mallinckrodt, AR); add 200 ml distilled water, washing down the neck of the flask and shake until solution is complete; add 50 ml 85% phosphoric acid (H_3PO_4—Baker & Adamson, A.C.S.) and mix thoroughly; add 100 ml concentrated hydrochloric acid (HCl—Baker & Adamson, A.C.S.) and mix thoroughly; fit a Liebig reflux condenser to the flask and boil gently for 10 hours (during this period the solution changes in color from a pale yellow to a deep green); remove the reflux condenser; add 150 gm lithium sulfate ($LiSO_4$—Baker & Adamson, Reagent) and 50 ml distilled water, washing down the neck of the flask; shake thoroughly until completely dissolved; add 10 drops bromine (Br—Baker & Adamson, A.C.S.) and boil for 15 minutes to remove the excess bromine; allow to cool to room temperature; dilute to 1000 ml with distilled water and filter through tightly packed glass wool into a brown glass-stoppered bottle. The reagent should show no tint of green and if kept well protected from dust will remain stable for months. A reagent which develops a green color in the course of time may be repurified by the addition of a few drops of bromine and boiling as before.

Technic

1. In a 10 ml graduated test tube place about 5 ml 0.85% sodium chloride (1).
2. Add 0.5 ml sample from an Ostwald-Folin pipette, thoroughly rinsing out the pipette by drawing in and expelling some of the mixture several times.
3. Dilute to 10 ml with 0.85% sodium chloride (1) and mix.
4. In an 18 × 150 mm Pyrex test tube place
   1 ml diluted sample
   5 ml distilled water
   4 ml 2.5 N sodium hydroxide (2) and mix.
5. Place in boiling water for 10 minutes.
6. Cool in running tap water for 3 minutes.
7. Quantitatively transfer, through a funnel and Whatman #40 paper into a 100 ml volumetric flask (sample)—the filtration should be carried out even if no precipitate is apparent.
8. In another 100 ml volumetric flask (reagent blank) place
   4 ml 2.5 N sodium hydroxide (2).
9. To each flask add about 50 ml distilled water and mix.
10. Add 3 ml phenol reagent (3), slowly and with constant agitation of the flask.
11. Dilute to volume with distilled water and mix thoroughly by inversion.

12. Allow to stand at room temperature for 10 minutes.
13. Measure the transmittancy of the sample against the reagent blank set at 100% transmission at a wave length of **700 mμ** and obtain the concentration of total protein from the calibration table.

CALIBRATION PROCEDURE

Special Apparatus

Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.

Reagents

2. **Sodium Hydroxide, Volumetric 2.5 N.** Same as for Test Procedure.
3. **Phenol Reagent (Folin-Ciocalteu).** Same as for Test Procedure.
4. **Hydrochloric Acid, Volumetric 0.1 N.** Add 10 ml concentrated hydrochloric acid (HCl—Baker & Adamson, A.C.S.) to 1000 ml distilled water and mix; standardize against 0.1 N alkali using phenolphthalein as indicator.
5. **Total Protein Standards**
   - (a) Tyrosine stock standard (1 ml contains 0.2 mg tyrosine). In a 100 ml volumetric flask place 20 mg l-tyrosine ($C_9H_{11}O_3N$—Pfanstiehl, C.P.), add about 85 ml 0.1 N hydrochloric acid (4); shake until dissolved and dilute to volume with same. Preserve with a few drops of xylene.
   - (b) Dilute standard (1 ml contains 0.05 mg tyrosine). In a 100 ml volumetric flask place 25 ml stock standard (5a) and dilute to volume with distilled water. Prepare just before use.
   - (c) Calibration standards. In a series of six accurately calibrated 100 ml volumetric flasks place respectively 0, 2.5, 5, 7.5, 10 and 12.5 ml of the dilute standard (5b) and proceed as follows. These standards represent concentrations of total protein equivalent to 0, 2.9, 5.8, 8.6, 11.5 and 14.4%. Prepare just before use.

Technic

1. To each calibration standard (5c) add 4 ml 2.5 N sodium hydroxide (2).
2. Proceed exactly as outlined in the Test Procedure, Steps 9–12 inclusive.
3. Set the zero concentration standard at 100% transmission at a wave length of **700 mμ** and record the transmittancy of the 2.9, 5.8, 8.6, 11.5 and 14.4% standards. The transmittancy of the 14.4% standard should be approximately 27%.
4. Plot the observed values on semi-log graph paper—the points fall on a straight line which passes through the origin (Calibration Curve P-52A).
5. Prepare a calibration table from the T-C values obtained from the plotted curve.

Method P-52b

DETERMINATION IN CEREBROSPINAL FLUID

SAMPLE

The sample should be clear and colorless—the presence of blood or heavy bacterial growth invalidates the results. The analysis should not be unduly delayed.

TEST PROCEDURE

Special Apparatus

Ostwald-Folin pipettes accurately calibrated "to deliver" 1 ml.

Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.

Reagents

2. **Sodium Hydroxide, Volumetric 2.5 N.** Same as for blood.
3. **Phenol Reagent (Folin-Ciocalteu).** Same as for blood.

Technic

Note: For concentrations of total protein less than 100 mg per 100 ml, use 5 ml cerebrospinal fluid and 1 ml distilled water in Step 1 and divide the values from the calibration table for cerebrospinal fluid by 5 to obtain the concentration of total protein in mg per 100 ml; for concentrations in excess of 600 mg per 100 ml, use 0.5 ml cerebrospinal fluid and 5.5 ml distilled water in Step 1 and multiply the values from the calibration table for cerebrospinal fluid by 2.

1. In an 18 × 150 mm Pyrex test tube place
   1 ml clear cerebrospinal fluid
   5 ml distilled water
   4 ml 2.5 N sodium hydroxide (2) and mix.
2. Place in boiling water for 10 minutes.
3. Cool in running tap water for 3 minutes.
4. Quantitatively transfer, through a funnel and Whatman #40 paper into a 100 ml volumetric flask (sample)—the filtration should be carried out even if no precipitate is apparent.
5. In another 100 ml volumetric flask (reagent blank) place
   4 ml 2.5 N sodium hydroxide (2).
6. To each flask add about 50 ml distilled water and mix.
7. Add 3 ml phenol reagent (3), slowly and with constant agitation of the flask.
8. Dilute to volume with distilled water and mix thoroughly by inversion.

9. Allow to stand at room temperature for 10 minutes.
10. Measure the transmittancy of the sample against the reagent blank set at 100% transmission at a wave length of **700** mμ and obtain the concentration of total protein from the calibration table for cerebrospinal fluid.

CALIBRATION PROCEDURE

This calibration procedure need not be carried out. A calibration curve (Calibration Curve P-52B) may be constructed by substituting the values 144, 288, 431, 575 and 719 mg per 100 ml for the transmittancy readings recorded for the 2.9, 5.8, 8.6, 11.5 and 14.4% concentrations in the calibration curve for blood.

PROCEDURE FOR USE WITH LINEAR-SCALE PHOTOELECTRIC COLORIMETERS (SHEARD-SANFORD TYPE)

TEST PROCEDURE

Exactly as outlined; set the reagent blank at 100 and use a red filter.

CALIBRATION PROCEDURE

Exactly as outlined—use a red filter; plot the observed readings on semi-log graph paper—the points fall on a straight line passing through the origin.

PROCEDURE FOR USE WITH LOGARITHMIC-SCALE PHOTOELECTRIC COLORIMETERS (KLETT-SUMMERSON TYPE)

TEST PROCEDURE

Exactly as outlined; set the reagent blank at 0 and use a red filter.

CALIBRATION PROCEDURE

Exactly as outlined—use a red filter; plot the observed readings on linear graph paper—the points fall on a straight line passing through the origin.

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NOTES

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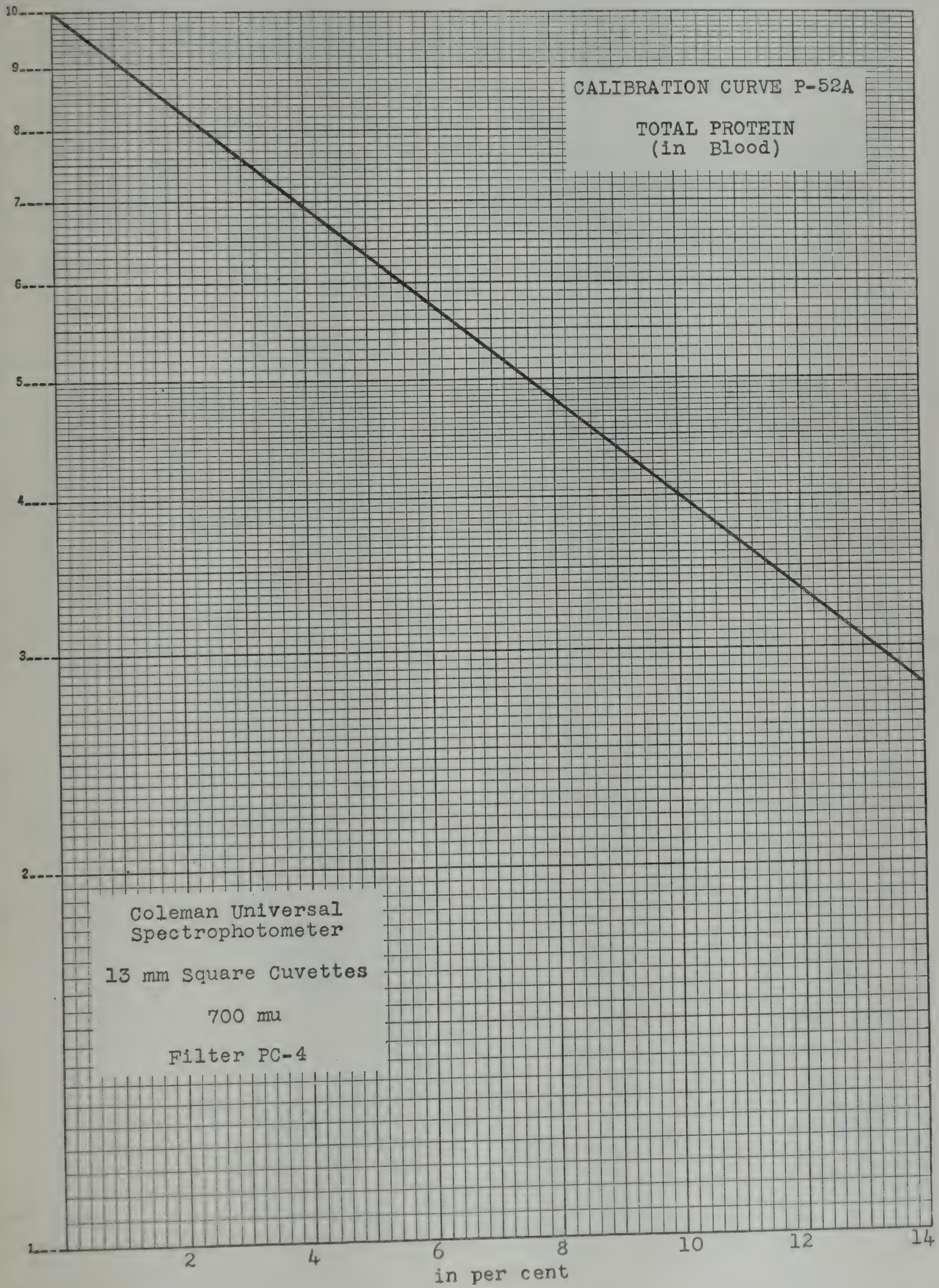
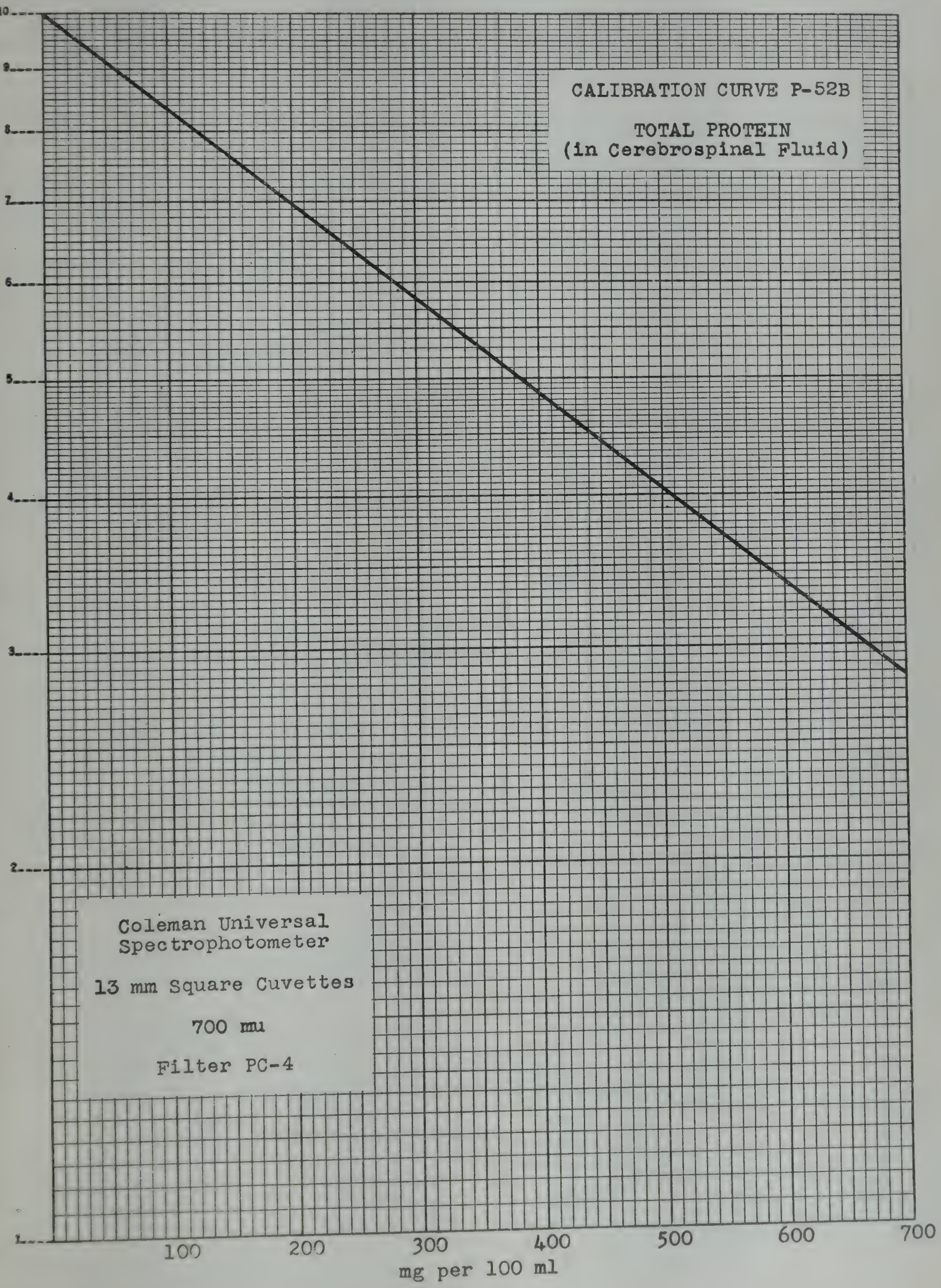

CALIBRATION CURVE P-52A
TOTAL PROTEIN
(in Blood)
Coleman Universal Spectrophotometer
13 mm Square Cuvettes
700 mu
Filter PC-4
10
9
8
7
6
5
4
3
2
1
2
4
6
8
10
12
14
in per cent

Meter Reading	0	1	2	3	4	5	6	7	8	9
0										
10										
20										
30										
40										
50										
60										
70										
80										
90										



CALIBRATION CURVE P-52B

TOTAL PROTEIN
(in Cerebrospinal Fluid)

Coleman Universal
Spectrophotometer

13 mm Square Cuvettes

700 mu

Filter PC-4

Meter Reading	0	1	2	3	4	5	6	7	8	9
0										
10										
20										
30										
40										
50										
60										
70										
80										
90										

PROTEIN, TOTAL

A spectrophotometric adaptation based on the method of Looney and Walsh (1939)

PRINCIPLE

Protein is precipitated by sulfosalicylic acid (Roche reaction), the suspension being stabilized by means of gum ghatti.

DETERMINATION IN CEREBROSPINAL FLUID

SAMPLE

The cerebrospinal fluid should be clear and colorless—the presence of blood or heavy bacterial growth invalidates the results. The analysis should not be unduly delayed.

TEST PROCEDURE

Special Apparatus

Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.

Reagents

1. **Gum Ghatti, 2% Aqueous.** Fill a 1000 ml cylinder to volume with distilled water; suspend, just below the surface, a cloth bag containing 20 gm soluble gum ghatti (Coleman & Bell); allow to stand for 24 hours; remove the bag and filter the solution through a coarse paper. Preserve with a few drops of xylene and store in refrigerator.
2. **Sulfosalicylic Acid, 5% Aqueous.** Dissolve 5 gm crystalline sulfosalicylic acid ($SO_3H \cdot C_6H_3 \cdot OH \cdot COOH$—Merck, Reagent) in iron-free distilled water and dilute to 100 ml. Only pure white crystals should be used and solutions showing a pinkish tinge should be discarded.

Technic

Note: For concentrations of protein in excess of 250 mg per 100 ml, use 1 ml sample and 4 ml distilled water in Step 1 and multiply the values obtained from the calibration table by 2.

1. In a test tube (sample) place
   2 ml clear cerebrospinal fluid
   3 ml distilled water and mix gently.
2. In another test tube (reagent blank) place
   5 ml distilled water.
3. To each tube add 1 ml 2% gum ghatti (1) and mix by gentle lateral whirling.
4. Add 4 ml 5% sulfosalicylic acid (2) and mix by gentle lateral whirling.
5. Allow to stand at room temperature for 10 minutes.
6. Mix gently and immediately measure the transmittancy of the sample against the reagent blank set at 100% transmission at a wave length of 660 mμ and obtain the concentration of total protein from the calibration table.

CALIBRATION PROCEDURE

Special Apparatus

Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.

Reagents

1. **Gum Ghatti, 2% Aqueous.** Same as for Test Procedure.
2. **Sulfosalicylic Acid, 5% Aqueous.** Same as for Test Procedure.
3. **Sodium Chloride, 0.85% Aqueous.** Dissolve 8.5 gm sodium chloride (NaCl—Merck, Special for Biological Work) in distilled water and dilute to 1000 ml.
4. **Total Protein Standards**
   (a) Protein stock standard. Determine the total protein in a well mixed sample of pooled human sera by the Kjeldahl procedure (Method P-51).
   (b) Dilute standard (1 ml contains 1 mg protein). In a 100 ml volumetric flask place a volume of pooled sera (4a) containing 100 mg protein and dilute to volume with 0.85% sodium chloride (3). Prepare just before use.
   (c) Calibration standards. In a series of seven 25 ml test tubes place respectively 0, 0.2, 0.6, 1, 2, 3 and 5 ml of the dilute standard (4b) and add 5, 4.8, 4.4, 4, 3, 2 and 0 ml distilled water respectively, bringing the volume in each tube to 5 ml. These standards represent concentrations of protein equivalent to 0, 10, 30, 50, 100, 150 and 250 mg per 100 ml sample. Prepare just before use and proceed immediately as follows.

Technic

1. Treat each calibration standard (4c) exactly as outlined in the Test Procedure, Steps 3–5 inclusive.
2. Mix gently and immediately set the zero concentration standard at 100% transmission at a wave length of 660 mμ and record the transmittancy of the 10, 30, 50, 100, 150 and 250 mg per cent standards. The transmittancy of the 250 mg standard should be approximately 22%.

3. Plot the observed values on semi-log graph paper—the points fall on a smooth curve which passes through the concentration axis slightly to the right of the origin (Calibration Curve P-53).
4. Prepare a calibration table from the T-C values obtained from the plotted curve.

Note: Whereas the calibration curves for all colorimetric procedures listed in this manual are interchangeable from one Coleman spectrophotometer to another and even from one model to another, the calibration curves for the nephelometric procedures are not interchangeable. Calibration curves must be prepared for every instrument.

PROCEDURE FOR USE WITH LINEAR-SCALE PHOTOELECTRIC COLORIMETERS (SHEARD-SANFORD TYPE)

TEST PROCEDURE

Exactly as outlined; set the reagent blank at 100 and use a red filter.

CALIBRATION PROCEDURE

Exactly as outlined—use a red filter; plot the observed readings on semi-log graph paper—the points fall on a smooth curve passing through the concentration axis slightly to the right of the origin.

PROCEDURE FOR USE WITH LOGARITHMIC-SCALE PHOTOELECTRIC COLORIMETERS (KLETT-SUMMERSON TYPE)

TEST PROCEDURE

Exactly as outlined; set the reagent blank at 0 and use a red filter.

CALIBRATION PROCEDURE

Exactly as outlined—use a red filter; plot the observed readings on linear graph paper—the points fall on a smooth curve passing through the concentration axis slightly to the right of the origin.

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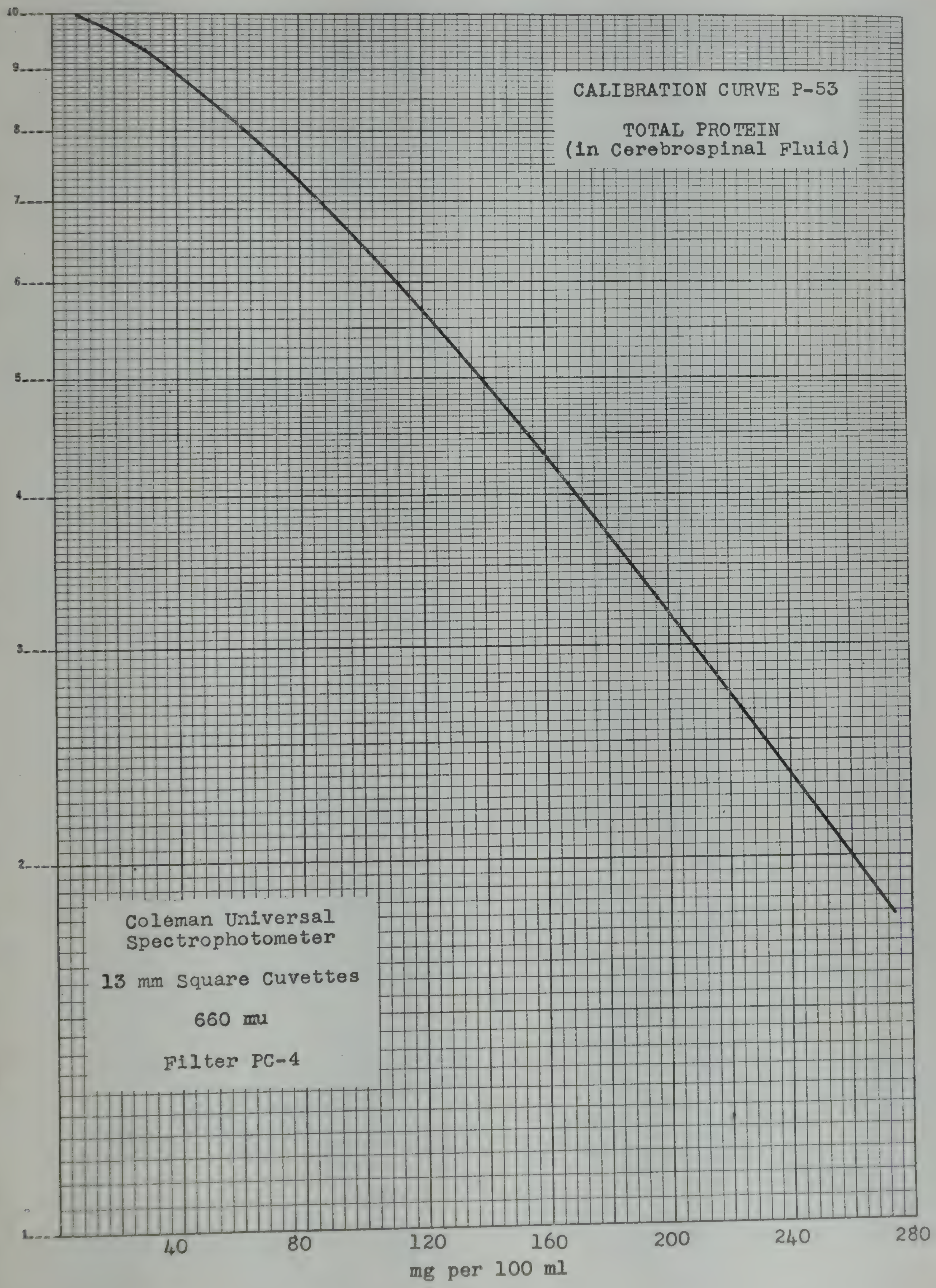
NOTES


CALIBRATION CURVE P-53
TOTAL PROTEIN
(in Cerebrospinal Fluid)
Coleman Universal Spectrophotometer
13 mm Square Cuvettes
660 mu
Filter PC-4
10
9
8
7
6
5
4
3
2
1
40
80
120
160
200
240
280
mg per 100 ml

Meter Reading	0	1	2	3	4	5	6	7	8	9
0										
10										
20										
30										
40										
50										
60										
70										
80										
90										

PROTEIN, TOTAL

A spectrophotometric adaptation based on the method of Exton (1923; 1925)

PRINCIPLE

Protein is precipitated by sulfosalicylic acid in the presence of sodium sulfate and bromphenol blue (Exton reaction), the suspension being stabilized by means of gum ghatti.

DETERMINATION IN URINE

SAMPLE

Aliquot of a 24-hour specimen. For samples showing gross bacterial contamination, add 10 gm infusorial earth (Reagent #1) to a 4 ounce aliquot and shake thoroughly before proceeding with Step 1 (the protein removed by adsorption is negligible).

TEST PROCEDURE

Special Apparatus

Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.

Reagents

1. **Infusorial Earth, Powder** (Baker & Adamson).
2. **Acetic Acid, Glacial** (CH_3COOH—Baker & Adamson, A.C.S.).
3. **Gum Ghatti, 2% Aqueous.** Fill a 1000 ml cylinder to volume with distilled water; suspend, just below the surface, a cloth bag containing 20 gm soluble gum ghatti (Coleman & Bell); allow to stand for 24 hours; remove the bag and filter the solution through a coarse paper. Preserve with a few drops of xylene and store in refrigerator.
4. **Exton Reagent, Modified.** In a 1500 ml beaker place about 800 ml distilled water; add 50 gm sulfosalicylic acid ($SO_3H \cdot C_6H_3 \cdot OH \cdot COOH$—Merck, Reagent) and 10 gm crystalline sodium sulfate ($Na_2SO_4 \cdot 10H_2O$—Baker & Adamson, Reagent); heat gently and stir until solution is complete; add 25 ml 0.04% bromphenol blue (a); mix and allow to cool; transfer to a 1000 ml mixing cylinder, dilute to volume with distilled water and filter. Stability indefinite.
   (a) Bromphenol blue, 0.04% aqueous; in a 250 ml volumetric flask place 102 mg of the water-soluble sodium salt of bromphenol blue ($C_{19}H_{10}Br_4O_5S$—LaMotte); dissolve in distilled water and dilute to volume. Preserve in resistant-glass bottle.

Technic

Note: For concentrations of protein in excess of 1000 mg per 100 ml, use 2.5 ml urine in Step 2 and 7.5 ml distilled water in Step 3 and multiply the values obtained from the calibration table by 2.

1. Filter a small portion of thoroughly mixed urine.
2. Place 5 ml clear filtered urine in a small flask and, if alkaline, add sufficient glacial acetic acid (2), drop by drop, to render distinctly acid.
3. Add 5 ml distilled water and mix by inversion.
4. In a test tube (sample) place
   1 ml diluted urine
   4 ml distilled water.
5. In another test tube (reagent blank) place
   5 ml distilled water.
6. To each tube add 1 ml 2% gum ghatti (3) and mix by gentle lateral whirling.
7. Add 5 ml Exton reagent (4) and mix by gentle lateral whirling.
8. Allow to stand at room temperature for 10 minutes.
9. Mix gently and immediately measure the transmittancy of the sample against the reagent blank set at 100% transmission at a wave length of 660 mμ and obtain the concentration of total protein from the calibration table.

Calculation

$$\frac{\text{Volume of 24-hour specimen}}{100} \times \text{Total protein in mg per 100 ml} = \text{Mg protein excreted in 24 hours}$$

CALIBRATION PROCEDURE

Special Apparatus

Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.

Reagents

3. **Gum Ghatti, 2% Aqueous.** Same as for Test Procedure.
4. **Exton Reagent, Modified.** Same as for Test Procedure.
5. **Sodium Chloride, 0.85% Aqueous.** Dissolve 8.5 gm sodium chloride (NaCl—Merck, Special for Biological Work) in distilled water and dilute to 1000 ml.
6. **Total Protein Standards**
   (a) Protein stock standard. Determine the total protein in a well mixed sample of pooled human sera by the Kjeldahl procedure (Method P-51).
   (b) Dilute standard (1 ml contains 1 mg protein). In a 100 ml volumetric flask place a volume of pooled sera (6a) containing 100 mg protein and dilute to volume with 0.85% sodium chloride (5). Prepare just before use.

(c) Calibration standards. In a series of seven 25 ml test tubes place respectively 0, 0.2, 0.6, 1, 2, 3 and 5 ml of the dilute standard (6b) and add 5, 4.8, 4.4, 4, 3, 2 and 0 ml distilled water respectively, bringing the volume in each tube to 5 ml. These standards represent concentrations of protein equivalent to 0, 40, 120, 200, 400, 600 and 1000 mg per 100 ml sample. Prepare just before use and proceed immediately as follows.

Technic

1. Treat each calibration standard (6c) exactly as outlined in the Test Procedure, Steps 6–8 inclusive.
2. Mix gently and immediately set the zero concentration standard at 100% transmission at a wave length of 660 mμ and record the transmittancy of the 40, 120, 200, 400, 600 and 1000 mg per cent standards. The transmittancy of the 1000 mg standard should be approximately 22%.
3. Plot the observed values on semi-log graph paper —the points fall on a smooth curve which passes through the concentration axis slightly to the right of the origin (Calibration Curve P-54).
4. Prepare a calibration table from the T-C values obtained from the plotted curve.

Note: Whereas the calibration curves for all colorimetric procedures listed in this manual are interchangeable from one Coleman spectrophotometer to another and even from one model to another, the calibration curves for the nephelometric procedures are not interchangeable. Calibration curves must be prepared for every instrument.

PROCEDURE FOR USE WITH LINEAR-SCALE PHOTOELECTRIC COLORIMETERS (SHEARD-SANFORD TYPE)

TEST PROCEDURE

Exactly as outlined; set the reagent blank at 100 and use a red filter.

CALIBRATION PROCEDURE

Exactly as outlined—use a red filter; plot the observed readings on semi-log graph paper—the points fall on a smooth curve passing through the concentration axis slightly to the right of the origin.

PROCEDURE FOR USE WITH LOGARITHMIC-SCALE PHOTOELECTRIC COLORIMETERS (KLETT-SUMMERSON TYPE)

TEST PROCEDURE

Exactly as outlined; set the reagent blank at 0 and use a red filter.

CALIBRATION PROCEDURE

Exactly as outlined—use a red filter; plot the observed readings on linear graph paper—the points fall on a smooth curve passing through the concentration axis slightly to the right of the origin.

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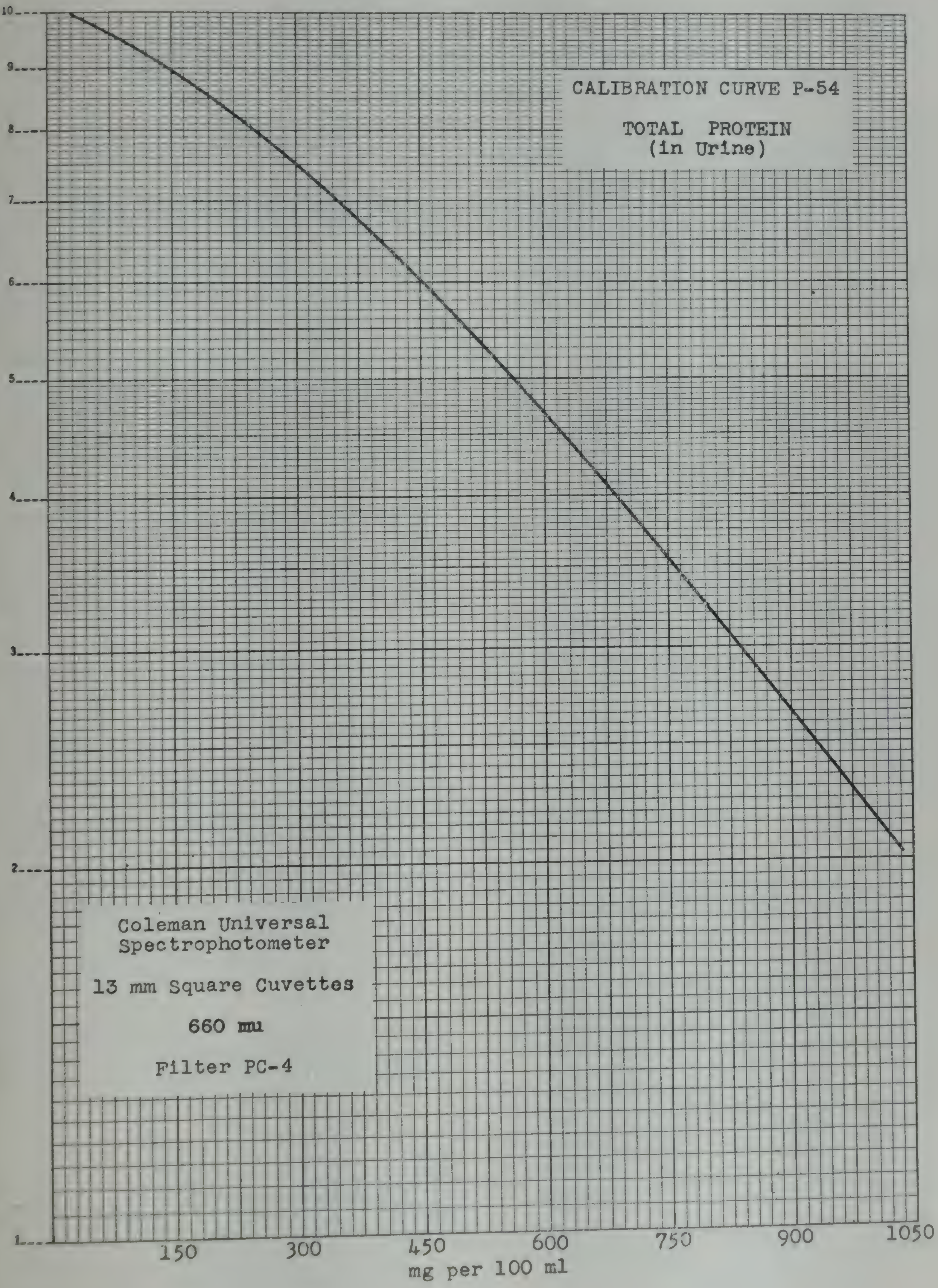
NOTES


CALIBRATION CURVE P-54
TOTAL PROTEIN
(in Urine)
Coleman Universal
Spectrophotometer
13 mm Square Cuvettes
660 mμ
Filter PC-4
10
9
8
7
6
5
4
3
2
1
150
300
450
600
750
900
1050
mg per 100 ml

Meter Reading	0	1	2	3	4	5	6	7	8	9
0										
10										
20										
30										
40										
50										
60										
70										
80										
90										

PYRUVIC ACID

A spectrophotometric adaptation based on the methods and modifications of Case (1932), Lu (1939), Bueding and Wortis (1940) and Friedemann and Haugen (1942; 1943)

PRINCIPLE

Pyruvic and other keto acids, glyoxals and aldehydes all form dinitrophenylhydrazones when treated with 2,4-dinitrophenylhydrazine. Initial extraction with xylene effects almost quantitative separation of the pyruvic acid hydrazone together with those of the glyoxals and aldehydes. Re-extraction with sodium carbonate separates practically all of the pyruvic hydrazone together with a slight amount of other acid hydrazones. Upon treatment of the carbonate phase with sodium hydroxide a brownish red color is developed (Bamberger reaction).

DETERMINATION IN WHOLE BLOOD

SAMPLE

Blood withdrawn with a minimum of stasis during post-absorptive state and after a rest period of at least 30 minutes—the tourniquet remaining on the arm only until the needle has entered the vein. The patient is instructed not to open and close the hand in order to distend the vein. Syringe and needle must be cold and dry. Use an accurately calibrated 2 ml tuberculin syringe fitted with a 21 gauge needle. Secure slightly more than 2 ml and then carefully free of any air bubbles by holding the syringe vertically and adjust volume to exactly 2 ml (tolerance ±1%). The blood is then expelled in a fine stream through the needle into 10 ml cold 10% trichloracetic acid (1) contained in a 15 ml centrifuge tube. Stopper the tube, shake well and refrigerate for at least 10 minutes before proceeding. Blood thus collected may be stored in refrigerator for as long as two days without demonstrable loss of pyruvic acid. In cases of ketosis, the sample should be allowed to stand overnight in the refrigerator to eliminate the slight effect of acetoacetic acid.

TEST PROCEDURE

Special Apparatus

Wright capillary pipettes.

Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.

Reagents

1. **Trichloracetic Acid, 10% Aqueous.** Dissolve 10 gm crystalline trichloracetic acid (CCl_3COOH—J. T. Baker, CP Analyzed) in distilled water and dilute to 100 ml. Prepare weekly. Preserve in Pyrex glass-stoppered bottle and store in refrigerator. Increased and variable blanks result from old solutions or from solutions which have stood for several hours at room temperature.
2. **2,4-Dinitrophenylhydrazine Reagent (Friedemann-Haugen).** Triturate 100 mg 2,4-dinitrophenylhydrazine ($(NO_2)_2C_6H_3NHNH_2$—Eastman #1866) in a mortar with successive small portions of 2 N hydrochloric acid (a) until 100 ml of the acid have been added. Filter through a small paper. Store in refrigerator.
    (a) Hydrochloric acid, volumetric 2 N; add 180 ml concentrated hydrochloric acid (HCl—Baker & Adamson, A.C.S.) to 1000 ml distilled water and mix well; titrate against standard 1 N alkali using phenolphthalein as indicator; adjust accordingly and re-titrate—20 ml of 2 N acid require 40 ml of 1 N alkali.
3. **Xylene.** ($C_6H_4(CH_3)_2$—Baker & Adamson, A.C.S.).
4. **Sodium Carbonate, 10% Aqueous.** Dissolve 10 gm anhydrous sodium carbonate (Na_2CO_3—Baker & Adamson, A.C.S.) in distilled water and dilute to 100 ml. Filter if not clear. Preserve in ceresin-lined bottle.
5. **Sodium Hydroxide, Volumetric 3 N.** Prepare by diluting slightly more than the required amount of concentrated carbonate-free sodium hydroxide solution (see Appendix) and standardize against 1 N acid using phenolphthalein as indicator—30 ml of 1 N acid require 10 ml of 3 N alkali. Preserve in ceresin-lined bottle.

Technic

Note: Measure all solutions accurately and carry analysis to completion as quickly as possible. No more than six determinations should be run at one time.

1. Centrifuge blood collection tube at 1500 rpm for 5 minutes.
2. Transfer 3 ml of the clear supernatant centrifugate to an 18 × 150 mm test tube (sample).
3. In a similar test tube (reagent blank) place 2.5 ml 10% trichloracetic acid (1) and 0.5 ml distilled water.
4. Warm both tubes in water bath at 25° C. for 10 minutes.
5. Add 1 ml 2,4-dinitrophenylhydrazine reagent (2) and mix.
6. Replace in water bath at 25° C. for exactly 5 minutes.
7. Add 3 ml xylene (3) and mix for 2 minutes by rapidly blowing a current of air (or nitrogen) through a Wright capillary pipette, placed so that

the tip of the pipette rests on the bottom of the tube.

8. Allow to stand until layers have separated—any aqueous globules that may adhere to the sides of the tube are removed by tapping or a rapid circular motion.
9. By means of the capillary pipette employed for aerating, carefully withdraw the lower aqueous layer and discard.
10. Add 6 ml 10% sodium carbonate (4) to the xylene phase and mix again for 2 minutes by rapidly aerating as in Step 7.
11. Allow to stand until layers have separated.
12. Using the capillary pipette withdraw as much as possible of the upper xylene layer and discard.
13. Quickly insert the tip of a transfer pipette, with the finger held over the top to prevent ingress of any of the xylene; gently blow through the pipette momentarily to expel any xylene that may have entered; withdraw 5 ml of the remaining aqueous phase, wiping the pipette free of adherent xylene, and place in a cuvette.
14. Add 2.5 ml 3 N sodium hydroxide (5) and mix immediately.
15. Allow to stand at room temperature for 10 minutes.
16. Measure the transmittancy of the sample against the reagent blank set at 100% transmission at a wave length of **520 mμ** and obtain the concentration of pyruvic acid from the calibration table.

CALIBRATION PROCEDURE

Special Apparatus

Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.
Graduated test tubes—10 ml in 0.1 ml; the 10 ml graduation must be accurately calibrated.
Wright capillary pipettes.

Reagents

1. **Trichloracetic Acid, 10% Aqueous.** Same as for Test Procedure.
2. **2,4-Dinitrophenylhydrazine Reagent (Friedemann-Haugen).** Same as for Test Procedure.
3. **Xylene.** Same as for Test Procedure.
4. **Sodium Carbonate, 10% Aqueous.** Same as for Test Procedure.
5. **Sodium Hydroxide, Volumetric 3 N.** Same as for Test Procedure.
6. **Sulfuric Acid, Volumetric 0.1 N.** Slowly add 3 ml concentrated sulfuric acid (H_2SO_4—Grasselli, A.C.S.) to 1000 ml distilled water and mix well; standardize against 0.1 N alkali using phenolphthalein as indicator.
7. **Pyruvic Acid Standards**
   - (a) Pyruvic acid stock standard, Lu (1 ml contains 1 mg pyruvic acid). In a 100 ml volumetric flask place 100 mg freshly distilled pyruvic acid ($CH_3COCOOH$—Reagent Chemicals, Special); dilute to volume with 0.1 N sulfuric acid (6). Preserve in refrigerator. Stability six months.
   - (b) Dilute standard (1 ml contains 0.05 mg pyruvic acid). In a 100 ml volumetric flask place 5 ml stock standard (7a) and dilute to volume with distilled water. Prepare just before use.
   - (c) Calibration standards. In a series of nine accurately calibrated 10 ml test tubes place respectively 0, 0.5, 1, 2, 4, 5, 6, 8 and 10 ml of the dilute standard (7b); add 10, 9.5, 9, 8, 6, 5, 4, 2 and 0 ml distilled water respectively, bringing the volume in each tube to 10 ml. These standards represent concentrations of pyruvic acid equivalent to 0, 0.3, 0.6, 1.2, 2.4, 3.0, 3.6, 4.8 and 6.0 mg per 100 ml sample. Prepare just before use.

Technic

1. Transfer 5 ml of each calibration standard (7c) to a correspondingly marked 25 ml volumetric flask.
2. Dilute to volume with cold 10% trichloracetic acid (1) and mix.
3. Transfer 3 ml from each flask to a correspondingly marked 18 × 150 mm test tube.
4. Proceed exactly as outlined in the Test Procedure, Steps 4–15 inclusive.
5. Set the zero concentration standard, which should show no pink tinge, at 100% transmission at a wave length of **520 mμ** and record the transmittancy of the 0.3, 0.6, 1.2, 2.4, 3.0, 3.6, 4.8 and 6.0 mg per cent standards. The transmittancy of the 6.0 mg standard should be approximately 32%.
6. Plot the observed values on semi-log graph paper —the points fall on a straight line which passes through the origin (Calibration Curve P-60).
7. Prepare a calibration table from the T-C values obtained from the plotted curve.

Method P-60b

DETERMINATION IN URINE

SAMPLE

Casual specimens of urine are collected in cold bottles; add 0.5 ml 20 N sulfuric acid (8) for each 100 ml urine; refrigerate immediately after collection. 24-hour specimens are collected in a cold bottle containing 5 ml 20 N sulfuric acid (8) and kept in the refrigerator. In specimens collected in this manner, the pyruvic acid content is not appreciably altered for at least 24 hours.

TEST PROCEDURE

Special Apparatus

As listed under the Test Procedure for blood.

Reagents

1. **Trichloracetic Acid, 10% Aqueous.** Same as for blood.
2. **2,4-Dinitrophenylhydrazine Reagent (Friedemann-Haugen).** Same as for blood.
3. **Xylene.** Same as for blood.
4. **Sodium Carbonate, 10% Aqueous.** Same as for blood.
5. **Sodium Hydroxide, Volumetric 3 N.** Same as for blood.
8. **Sulfuric Acid, Volumetric 20 N.** Slowly and with constant stirring, add 600 ml concentrated sulfuric acid (H_2SO_4—Grasselli, A.C.S.) to 400 ml distilled water in a 1500 ml beaker; allow to cool and make up volume to 1000 ml with distilled water; again allow to cool; dilute 1 ml of this approximately 20 N solution to 200 ml; titrate against standard 0.1 N alkali using phenolphthalein as indicator; adjust accordingly and retitrate—20 ml of a 1:200 dilution of 20 N acid require 20 ml of 0.1 N alkali. Preserve in glass-stoppered bottle.
9. **Lloyd's Reagent (Lilly).**

Technic

Note: Care in measurement of the solutions and especially accurate timing is essential in urine determinations. No more than six determinations should be run at one time.

1. To 10 ml urine add approximately 0.75 gm (not more than 1 gm) Lloyd's reagent (9).
2. Shake thoroughly and filter immediately.
3. Add 2 ml filtered urine to 10 ml cold 10% trichloracetic acid (1) contained in a 15 ml centrifuge tube.
4. Stopper the tube, shake well and allow to stand in refrigerator for at least 10 minutes (this dilute sample may be kept for at least 24 hours in the refrigerator with no change in the pyruvic acid content).
5. Centrifuge (if necessary) at 1500 rpm for 5 minutes.
6. Proceed exactly as outlined in the Test Procedure for blood, Steps 3–15 inclusive.
7. Measure the transmittancy of the sample against the reagent blank set at 100% transmission at a wave length of **520** mμ and obtain the concentration of pyruvic acid in mg per 100 ml urine directly from the calibration table for blood.

PROCEDURE FOR USE WITH LINEAR-SCALE PHOTOELECTRIC COLORIMETERS (SHEARD-SANFORD TYPE)

TEST PROCEDURE

Exactly as outlined; set the reagent blank at 100 and use a green filter.

CALIBRATION PROCEDURE

Exactly as outlined—use a green filter; plot the observed readings on semi-log graph paper—the points fall on a straight line passing through the origin.

PROCEDURE FOR USE WITH LOGARITHMIC-SCALE PHOTOELECTRIC COLORIMETERS (KLETT-SUMMERSON TYPE)

TEST PROCEDURE

Exactly as outlined; set the reagent blank at 0 and use a green filter.

CALIBRATION PROCEDURE

Exactly as outlined—use a green filter; plot the observed readings on linear graph paper—the points fall on a straight line passing through the origin.

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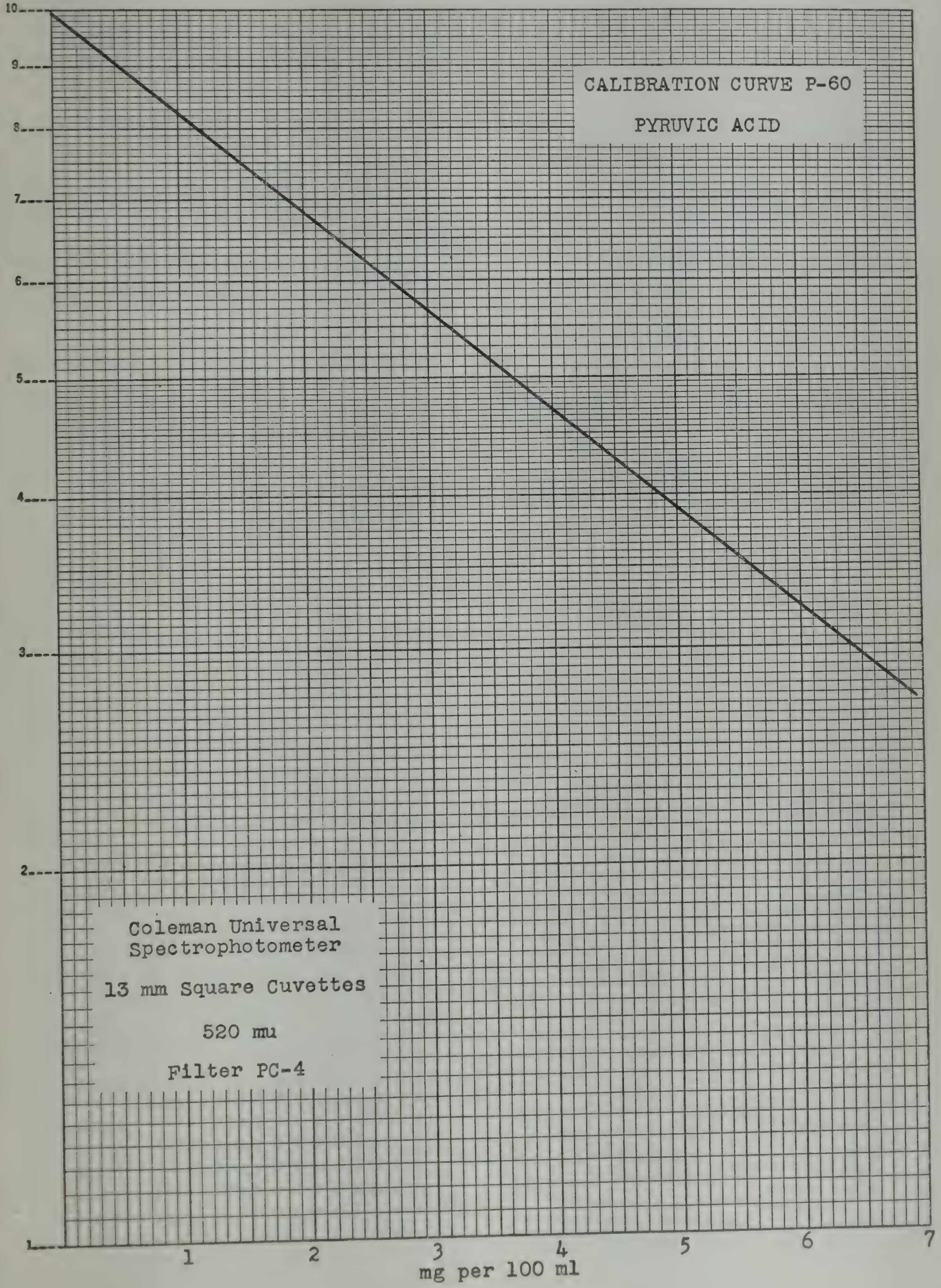
NOTES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mg per 100 ml

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SALICYLIC ACID

A spectrophotometric adaptation based on the methods and modifications of
Coburn (1943) and Brodie, Udenfriend and Coburn (1944)

PRINCIPLE

Free salicylic acid is extracted from acidified plasma or serum by means of ethylene chloride. Upon treatment with ferric nitrate, the salicylate is returned to the aqueous phase as a reddish-purple iron complex. This method measures the free salicylic acid only and is not applicable to the determination in urine in which the total salicylic acid is present in both the free and conjugated forms.

DETERMINATION IN PLASMA OR SERUM

SAMPLE

Plasma or serum from blood withdrawn at any time of the day, but preferably obtained during the post-absorptive state. Anticoagulant for plasma—potassium oxalate. When separated from the red cells, plasma or serum may be kept in the refrigerator for several days without loss of salicylic acid.

TEST PROCEDURE

Special Apparatus

Graduated centrifuge tubes, glass-stoppered, Pyrex, 50 ml; the 20 ml graduation must be accurately calibrated.

Ostwald-Folin pipettes accurately calibrated "to deliver" 2 ml.

Capillary pipettes fitted with rubber bulbs.

Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.

Reagents

1. **Ethylene Chloride** ($ClCH_2 \cdot CH_2Cl$—Eastman #132).
2. **Hydrochloric Acid, Volumetric 6 N.** Slowly add 500 ml concentrated hydrochloric acid (HCl—Baker & Adamson, A.C.S.) to 500 ml distilled water and mix; allow to cool; dilute 10 ml of this approximately 6 N solution to 100 ml; titrate against standard 0.5 N alkali using phenolphthalein as indicator; adjust accordingly and re-titrate—20 ml of a 1:10 dilution of 6 N acid require 24 ml of 0.5 N alkali. Preserve in a glass-stoppered bottle.
3. **Ferric Nitrate Reagent, Dilute.** Just before use dilute 1 ml concentrated ferric nitrate reagent (a) to 40 ml with distilled water and mix.
    (a) Ferric nitrate reagent, concentrated (Brodie-Udenfriend-Coburn); in a 100 ml mixing cylinder place 1 gm crystalline ferric nitrate ($Fe(NO_3)_3 \cdot 9H_2O$—Baker & Adamson, A.C.S.); add 95 ml volumetric 0.07 N nitric acid (i); shake until dissolved and dilute to volume with same.
        (i) Nitric acid, volumetric 0.07 N; add 5 ml concentrated nitric acid (HNO_3—Grasselli, A.C.S.) to 1000 ml distilled water and mix; titrate against standard 0.1 N alkali using phenolphthalein as indicator; adjust accordingly and re-titrate—20 ml of 0.07 N acid require 14 ml of 0.1 N alkali.

Technic

Note: For concentrations of salicylic acid less than 10 mg per 100 ml, use 2 ml sample in Step 2 and divide the values obtained from the calibration table by 2; for concentrations in excess of 50 mg per 100 ml, use 0.5 ml sample and 1.5 ml distilled water in Step 2 and multiply the values by 2.

1. In a 50 ml Pyrex glass-stoppered centrifuge tube place
    30 ml ethylene chloride (1), measured from a burette
    0.5 ml 6 N hydrochloric acid (2) and mix gently.
2. Add 1 ml sample from an Ostwald-Folin pipette, drop by drop, while constantly agitating the tube and 1 ml distilled water.
3. Stopper the tube and shake vigorously for 5 minutes.
4. Centrifuge the stoppered tube at 1500 rpm for 5 minutes.
5. By means of a capillary pipette fitted with a rubber bulb, remove the supernatant aqueous phase and discard.
6. Transfer exactly 20 ml of the ethylene chloride phase to a dry 50 ml graduated Pyrex glass-stoppered tube, by means of a pipette fitted with a rubber bulb.
7. Add 10 ml dilute ferric nitrate reagent (3).
8. Stopper the tube and shake vigorously for 5 minutes.
9. Centrifuge the stoppered tube at 1500 rpm for 3 minutes.
10. In a cuvette (sample) place 10 ml clear centrifugate.
11. In another cuvette (reagent blank) place 10 ml dilute ferric nitrate reagent (3).
12. Measure the transmittancy of the sample against the reagent blank set at 100% transmission at a wave length of 535 $m\mu$ and obtain the concentration of salicylic acid from the calibration table.

CALIBRATION PROCEDURE

Special Apparatus

Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.

Graduated centrifuge tubes, glass-stoppered, Pyrex, 50 ml; the 20 ml graduation must be accurately calibrated.

Capillary pipettes fitted with rubber bulbs.

Reagents

1. **Ethylene Chloride.** Same as for Test Procedure.
2. **Hydrochloric Acid, Volumetric 6 N.** Same as for Test Procedure.
3. **Ferric Nitrate Reagent, Dilute.** Same as for Test Procedure.
4. **Salicylic Acid Standards**
   (a) Salicylic acid stock standard (1 ml contains 1 mg salicylic acid). In a 100 ml volumetric flask place 116 mg sodium salicylate ($NaC_7H_5O_3$—Mallinckrodt, AR); add approximately 80 ml distilled water; shake until dissolved and dilute to volume. Preserve in refrigerator. Stability indefinite.
   (b) Dilute standard (1 ml contains 0.25 mg salicylic acid). In a 100 ml volumetric flask place 25 ml stock standard (4a) and dilute to volume with distilled water. Prepare just before use.
   (c) Calibration standards. In a series of six accurately calibrated 10 ml volumetric flasks place respectively 0, 2, 4, 6, 8 and 10 ml of the dilute standard (4b) and dilute to volume with distilled water. These standards represent concentrations of salicylic acid equivalent to 0, 10, 20, 30, 40 and 50 mg per 100 ml sample. Prepare just before use.

Technic

1. In each of a series of six 50 ml Pyrex glass-stoppered centrifuge tubes place
   30 ml ethylene chloride (1)
   0.5 ml 6 N hydrochloric acid (2) and mix gently.
2. Transfer 2 ml of each calibration standard (4c) to the correspondingly marked tube.
3. Proceed exactly as outlined in the Test Procedure, Steps 3–9 inclusive.
4. Transfer 10 ml of each clear centrifugate to a correspondingly marked cuvette.
5. Set the zero concentration standard at 100% transmission at a wave length of **535 mμ** and record the transmittancy of the 10, 20, 30, 40 and 50 mg per cent standards. The transmittancy of the 50 mg standard should be approximately 19%.
6. Plot the observed values on semi-log graph paper—the points fall on a straight line which passes through the origin (Calibration Curve S-10).
7. Prepare a calibration table from the T-C values obtained from the plotted curve.

CONVERSION FACTOR

Salicylic acid in mg per 100 ml × 1.16 = Sodium salicylate in mg per 100 ml.

◆

PROCEDURE FOR USE WITH LINEAR-SCALE PHOTOELECTRIC COLORIMETERS (SHEARD-SANFORD TYPE)

TEST PROCEDURE

Exactly as outlined; set the reagent blank at 100 and use a green filter.

CALIBRATION PROCEDURE

Exactly as outlined—use a green filter; plot the observed readings on semi-log graph paper—the points fall on a straight line passing through the origin.

◆

PROCEDURE FOR USE WITH LOGARITHMIC-SCALE PHOTOELECTRIC COLORIMETERS (KLETT-SUMMERSON TYPE)

TEST PROCEDURE

Exactly as outlined; set the reagent blank at 0 and use a green filter.

CALIBRATION PROCEDURE

Exactly as outlined—use a green filter; plot the observed readings on linear graph paper—the points fall on a straight line passing through the origin.

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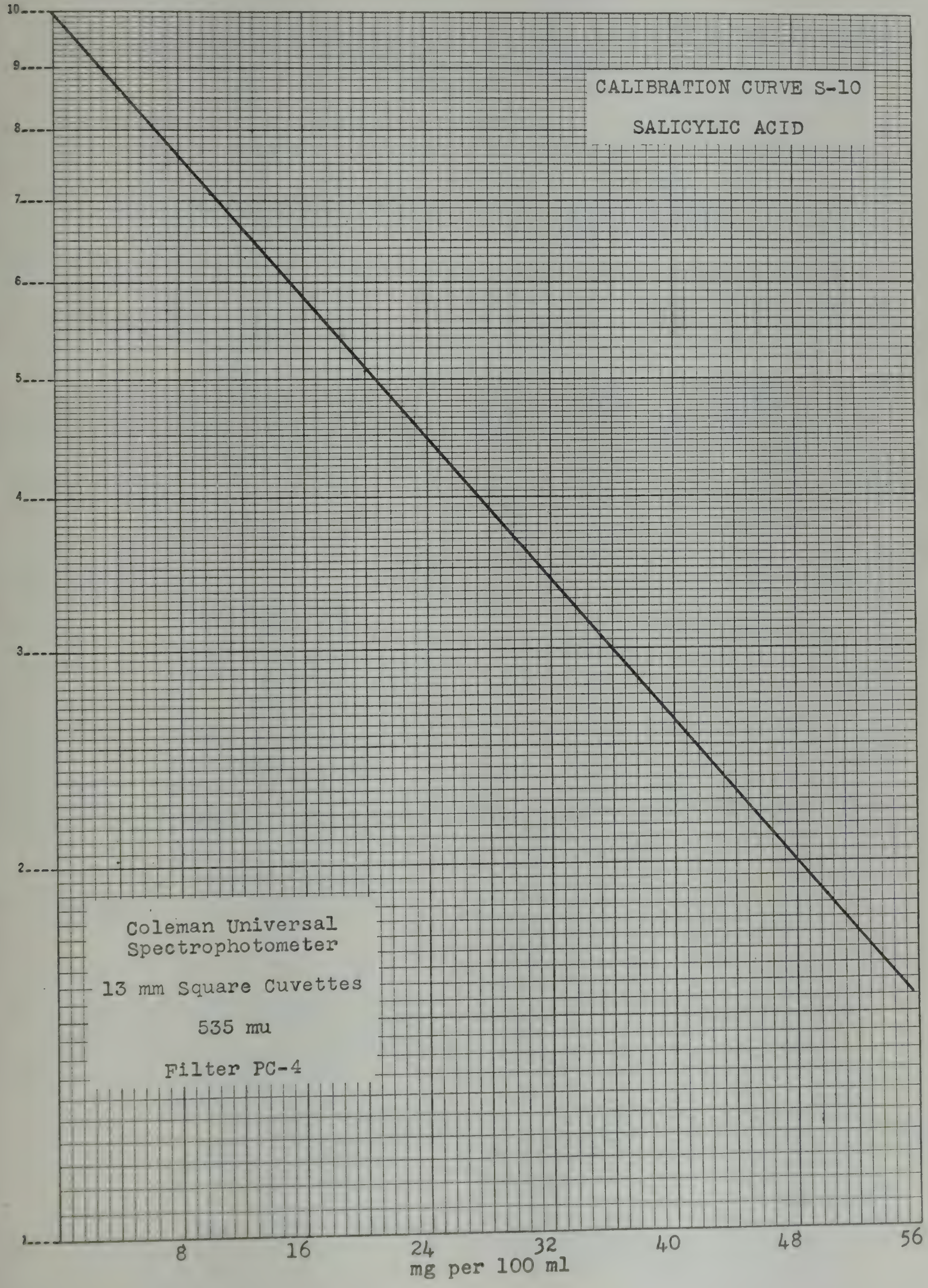
◆


CALIBRATION CURVE S-10
SALICYLIC ACID
Coleman Universal Spectrophotometer
13 mm Square Cuvettes
535 mu
Filter PC-4
10
9
8
7
6
5
4
3
2
1
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16
24
32
40
48
56
mg per 100 ml

Meter Reading	0	1	2	3	4	5	6	7	8	9
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SODIUM

A spectrophotometric adaptation based on the methods and modifications of Barber and Kolthoff (1928), Butler and Tuthill (1931) and Hoffman and Osgood (1938)

PRINCIPLE

Sodium is precipitated as uranium zinc sodium acetate. The yellow color of the aqueous solution of this salt is utilized for its quantitative determination. Ammonium thiocyanate is used to stabilize the color against temperature changes.

DETERMINATION IN SERUM

SAMPLE

Serum from blood withdrawn during post-absorptive state. The serum may be separated from the clot any time within 24 hours after collection. Analyses should not be made on less than 0.2 ml serum or its equivalent concentration of sodium.

TEST PROCEDURE

Special Apparatus

Ostwald-Folin pipettes accurately calibrated "to deliver" 0.5 ml.

Capillary-tipped pipettes with attached tubing and mouthpiece—volume greater than 2 ml.

Filtering assembly—consists of a 25 ml Pyrex micro filtering flask; a one-hole #0 rubber stopper; a 2 ml Pyrex micro filtering funnel with a fritted disc (porosity F) and rubber tubing to attach to water pump. Two assemblies are required for each determination.

Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.

Note: Glassware must be thoroughly cleaned with hot dilute nitric acid, rinsed with distilled water and dried before use—dichromate-sulfuric acid cleaning solution should not be used. Precautions should be taken to prevent contamination of all glassware and reagents by ammonia vapor.

Reagents

1. **Trichloracetic Acid, 20% Aqueous.** Dilute 20 ml concentrated trichloracetic acid (a) to 100 ml with distilled water. Prepare fresh monthly. Preserve in Pyrex glass-stoppered bottle.
    - (a) Trichloracetic acid, concentrated; dissolve 100 gm crystalline trichloracetic acid (CCl_3COOH—J. T. Baker, CP Analyzed) in distilled water and dilute to 100 ml. Preserve in Pyrex glass-stoppered bottle. Stability indefinite.
2. **Uranium Zinc Acetate Reagent (Barber-Kolthoff).** Prepare component solutions A and B as described below. Pour all of hot solution A into a 1500 ml beaker; immediately add all of hot solution B; stir thoroughly; allow to cool to room temperature, keeping beaker covered; add 0.2 gm uranium zinc sodium acetate ($(UO_2)_3ZnNa(C_2H_3O_2)_9 \cdot 6H_2O$—Reagent Chemicals, Special); stir thoroughly and allow to stand overnight; stir again and transfer fluid and precipitate to a brown bottle. Before use filter a sufficient amount of the reagent through a Whatman #40 paper. THIS REAGENT IS EXTREMELY POISONOUS.
    - A. Uranium acetate solution; in a 1000 ml beaker place 80 gm uranium acetate ($UO_2(C_2H_3O_2) \cdot 2H_2O$—Mallinckrodt, AR); add 48 ml 30% acetic acid (a) and 392 ml distilled water; heat on a steam bath, stirring frequently, until solution is complete; keep beaker covered with a large watch crystal to minimize evaporation.
        - (a) Acetic acid, 30% aqueous; dilute 30 ml glacial acetic acid (CH_3COOH—Baker & Adamson, A.C.S.) to 100 ml with distilled water.
    - B. Zinc acetate solution; in a 1000 ml beaker place 220 gm zinc acetate ($Zn(C_2H_3O_2)_2 \cdot 2H_2O$—Mallinckrodt, AR); add 24 ml 30% acetic acid (same as (a) above) and 276 ml distilled water; heat on a steam bath, stirring frequently, until solution is complete; keep beaker covered with a large watch crystal to minimize evaporation.
3. **Acetic Acid-Alcohol Wash Solution (Hoffman).** Mix 75 ml glacial acetic acid (CH_3COOH—Baker & Adamson, A.C.S.) with 425 ml 95% ethyl alcohol (C_2H_5OH—U. S. Industrial Chemicals, U.S.P.); shake with an excess of uranium zinc sodium acetate ($(UO_2)_3ZnNa(C_2H_3O_2)_9 \cdot 6H_2O$—Reagent Chemicals, Special) until the solution is saturated; transfer to a brown bottle without removing the excess triple salt. Before use filter a sufficient amount of the solution.
4. **Ether, Anhydrous.** ($(C_2H_5)_2O$—Mallinckrodt, A.C.S.). U.S.P. or ordinary C.P. ethers are not satisfactory.
5. **Ammonium Thiocyanate, Volumetric 0.1 N.** Dissolve 4 gm crystalline ammonium thiocyanate (NH_4CNS—Baker & Adamson, A.C.S.) in 500 ml distilled water; titrate against standard 0.1 N silver nitrate using ferric alum as indicator; adjust accordingly and re-titrate—20 ml of 0.1 N silver nitrate require 20 ml of 0.1 N ammonium thiocyanate. Prepare fresh monthly.

Technic

Note: Extreme accuracy in measurements and meticulous attention to detail are essential.

1. In a 15 ml centrifuge tube (sample) place
   3.5 ml distilled water
   0.5 ml serum from an Ostwald-Folin pipette
   1.0 ml 20% trichloracetic acid (1).
2. In another 15 ml centrifuge tube (reagent blank) place
   4.0 ml distilled water
   1.0 ml 20% trichloracetic acid (1).
3. Mix contents of both tubes thoroughly and allow to stand for 5 minutes.
4. Centrifuge at 2000 rpm for 10 minutes.
5. From each tube remove 2 ml supernatant fluid by means of capillary-tipped pipettes fitted with tubing and mouthpiece and transfer to correspondingly marked 20 ml beakers.
6. To each beaker add 11 ml uranium zinc acetate reagent (2) and stir vigorously with small glass rods for 4 minutes.
7. Without removing the stirring rods from the beakers, allow to stand for 15 minutes.
8. During this interval set up two similar filtering assemblies as follows: Insert the stem of a dry 2 ml fritted (porosity F) Pyrex funnel through a one-hole #0 rubber stopper; fit the stopper into a 25 ml Pyrex filtering flask and attach to suction pump (if only one pump is available a Y-connection will enable both aspirations to be made simultaneously).
9. Wash off the rods with not more than 1 ml of the uranium zinc acetate reagent and then remove from the beakers.
10. Start the suction and slowly transfer the contents of the beakers quantitatively to the correspondingly marked funnels—by pouring slowly and with constant suction the entire amount of fluid can be transferred to the funnel in one operation.
11. Wash out the beakers carefully with 2 ml uranium zinc acetate reagent (2), transferring the washings to funnels—if the glassware has been properly cleaned, none of the precipitate should adhere to the beaker, otherwise scrubbing with a rubber policeman is necessary.
12. Repeat Step 11.
13. When the funnels have been sucked empty add 2 ml acetic acid-alcohol wash solution (3) to each and again suck empty.
14. Add 5 ml ether (4) to each funnel and suck dry.
15. Repeat Step 14.
16. Continue aspirating until the last trace of ether has evaporated.
17. Remove funnels and stoppers from flasks and insert into another set of dry 25 ml filtering flasks—discard contents of original flasks.
18. To each funnel slowly add exactly 10 ml 0.1 N ammonium thiocyanate (5) and suck dry—2 minutes should suffice.
19. Transfer the contents of the filtering flasks to correspondingly marked cuvettes.
20. Measure the transmittancy of the sample against the reagent blank set at 100% transmission at a wave length of 400 mμ and obtain the concentration of sodium from the calibration table.

CALIBRATION PROCEDURE

Special Apparatus

Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.

Reagents

5. **Ammonium Thiocyanate, Volumetric 0.1 N.** Same as for Test Procedure.
6. **Sodium Standards**
   (a) Sodium stock standard (1 ml contains 6.15 mg uranium zinc sodium acetate). In a 100 ml volumetric flask place 615 mg uranium zinc sodium acetate ($(UO_2)_3ZnNa(C_2H_3O_2)_9 \cdot 6H_2O$—Reagent Chemicals, Special); add approximately 50 ml 0.1 N ammonium thiocyanate (5) and shake until dissolved; dilute to volume with same. Prepare shortly before use as the color slowly fades.
   (b) Calibration standards. In a series of eleven accurately calibrated 10 ml volumetric flasks place respectively 0, 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10 ml of the stock standard (6a) and dilute to volume with 0.1 N ammonium thiocyanate (5). These standards represent concentrations of sodium equivalent to 0, 20, 40, 60, 80, 100, 120, 140, 160, 180 and 200 meq per liter sample. Prepare just before use and proceed immediately as follows.

Technic

1. Set the zero concentration standard at 100% transmission at a wave length of 400 mμ and record the transmittancy of the 20, 40, 60, 80, 100, 120, 140, 160, 180 and 200 meq per liter standards (6b). The transmittancy of the 200 meq standard should be approximately 25%.
2. Plot the observed values on semi-log graph paper—the points fall on a smooth curve which passes through the origin (Calibration Curve S-15).
3. Make an enlargement of the above calibration curve by spreading the number of concentration divisions between the 130 and 150 meq per liter concentrations (Calibration Curve S-15A). This enables a more accurate determination of the values within the normal range.
4. Prepare a calibration table from the T-C values obtained from the plotted curves.

CONVERSION FACTOR

Sodium in milliequivalents per liter × 2.3 = Sodium in mg per 100 ml

PROCEDURE FOR USE WITH LINEAR-SCALE PHOTOELECTRIC COLORIMETERS (SHEARD-SANFORD TYPE)

TEST PROCEDURE

Exactly as outlined; set the reagent blank at 100 and use a blue filter.

CALIBRATION PROCEDURE

Exactly as outlined—use a blue filter; plot the observed readings on semi-log graph paper—the points fall on a smooth curve passing through the origin.

PROCEDURE FOR USE WITH LOGARITHMIC-SCALE PHOTOELECTRIC COLORIMETERS (KLETT-SUMMERSON TYPE)

TEST PROCEDURE

Exactly as outlined; set the reagent blank at 0 and use a blue filter.

CALIBRATION PROCEDURE

Exactly as outlined—use a blue filter; plot the observed readings on linear graph paper—the points fall on a smooth curve passing through the origin.

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NOTES

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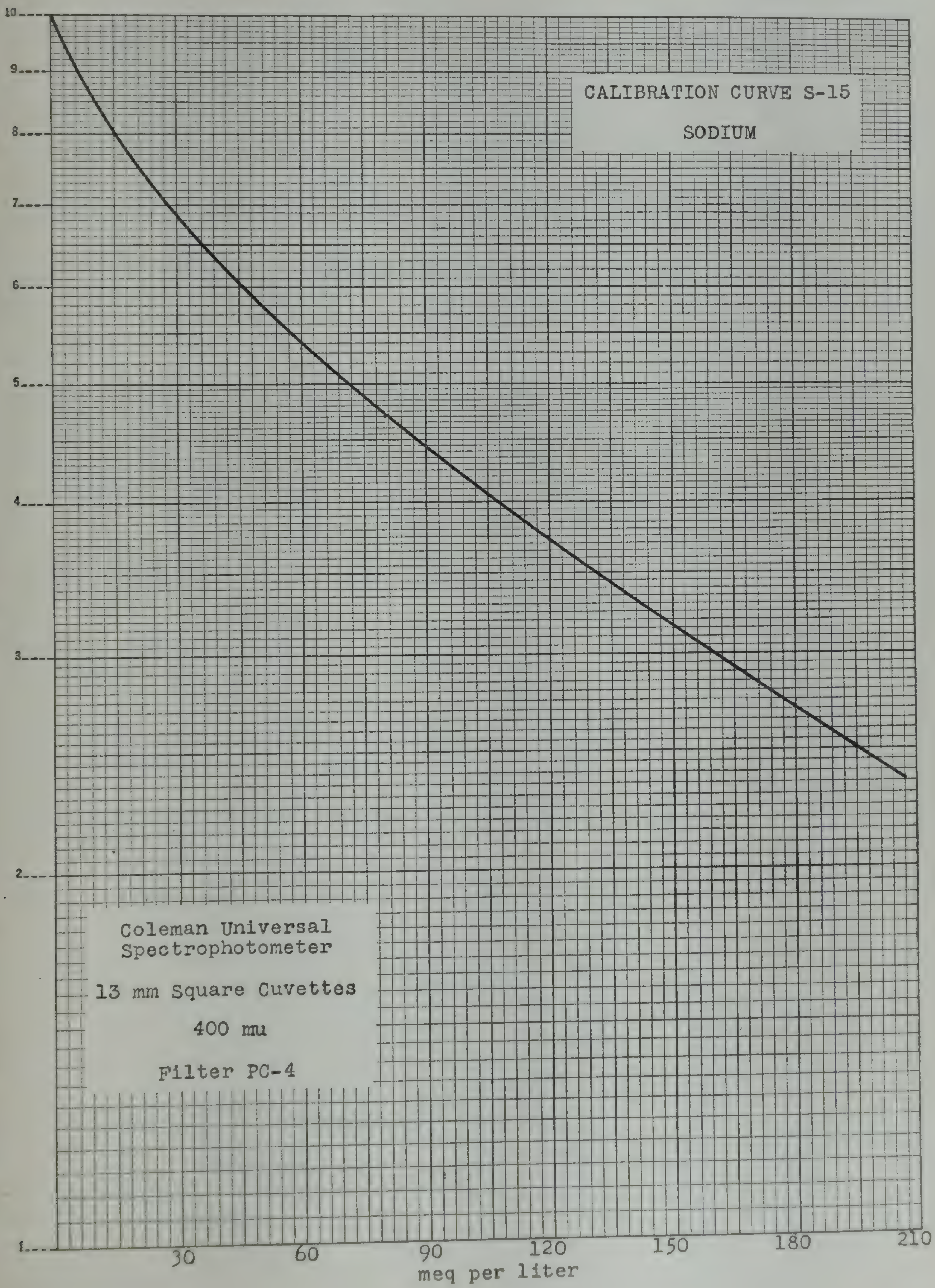
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meq per liter

Meter Reading	0	1	2	3	4	5	6	7	8	9
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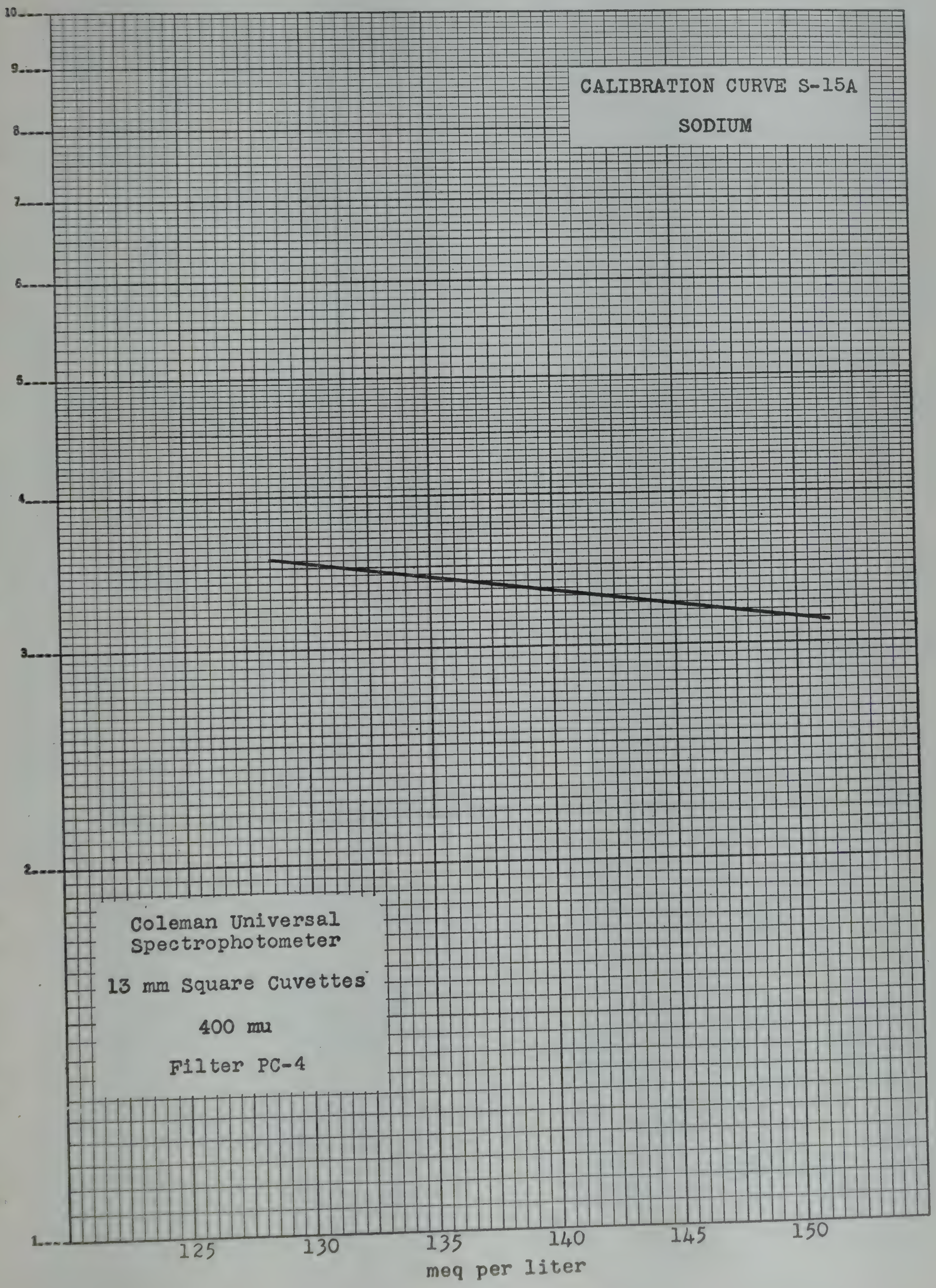
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Filter PC-4
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140
145
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meq per liter

Meter Reading	0	1	2	3	4	5	6	7	8	9
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SULFANILAMIDE, FREE

A spectrophotometric adaptation based on the methods and modifications of Fuller (1937), Marshall (1937), Marshall, Emerson and Cutting (1937), Hecht (1938), Marshall and Litchfield (1938), Bratton and Marshall (1939) and Holbourn and Pattle (1943)

PRINCIPLE

The free primary aryl amino group of sulfanilamide in a protein-free trichloracetic acid filtrate of hemolyzed whole blood, plasma or serum is diazotized with nitrous acid, the excess nitrite being destroyed by the addition of ammonium sulfamate. The diazotized sulfonamide is coupled with N-(1-naphthyl)-ethylenediamine dihydrochloride to form a purplish-red azo dye (Bratton-Marshall reaction).

DETERMINATION IN WHOLE BLOOD, PLASMA OR SERUM

SAMPLE

Blood withdrawn at any time of the day. Anticoagulant—potassium oxalate; an excess must be avoided. Analyses preferably made on whole blood; plasma contains less sulfanilamide than whole blood. Procaine and most of the antisyphilitic arsenicals interfere by producing similar colors.

TEST PROCEDURE

Special Apparatus

Ostwald-Folin pipettes accurately calibrated "to contain" 1 ml.

Graduated test tubes—25 ml in 0.5 ml; the 25 ml graduation must be accurately calibrated.

Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.

Reagents.

1. **Trichloracetic Acid, 15% Aqueous.** Dilute 15 ml concentrated trichloracetic acid (a) to 100 ml with distilled water. Preserve in glass-stoppered bottle. Prepare fresh monthly.
    (a) Trichloracetic acid, concentrated; dissolve 100 gm crystalline trichloracetic acid (CCl_3-COOH—J. T. Baker, CP Analyzed) in distilled water and dilute to 100 ml. Preserve in Pyrex glass-stoppered bottle. Stability indefinite.
2. **Trichloracetic Acid, 2.4% Aqueous.** Dilute 16 ml 15% trichloracetic acid (1) to 100 ml with distilled water. Preserve in refrigerator. Prepare fresh weekly.
3. **Sodium Nitrite, 0.1% Aqueous.** Dilute 1 ml 10% sodium nitrite (a) to 100 ml with distilled water. Prepare fresh daily and preserve in a brown glass bottle.
    (a) Sodium nitrite, 10% aqueous; dissolve 10 gm granular sodium nitrite ($NaNO_2$—Mallinckrodt, A.C.S.) in distilled water and dilute to 100 ml. Preserve in refrigerator.
4. **Ammonium Sulfamate, 0.5% Aqueous.** Dissolve 0.5 gm crystalline ammonium sulfamate ($NH_2SO_3NH_4$—Reagent Chemicals, Special) in distilled water and dilute to 100 ml. Stable indefinitely; discard if mold-growth occurs.
5. **N-(1-Naphthyl)-Ethylenediamine Dihydrochloride, 0.1% Aqueous (Bratton-Marshall).** Dissolve 100 mg N-(1-naphthyl)-ethylenediamine dihydrochloride ($C_{10}H_7HNCH_2CH_2NH_2 \cdot 2HCl$—Eastman #4835) in 100 ml distilled water. Preserve in a brown bottle in refrigerator. Stability about one month. Discard when a brownish color appears.

Technic

Note: If total sulfanilamide (Method S-23a) is to be determined on the same sample, reserve a portion of the trichloracetic acid filtrate in Step 5.

For concentrations of free sulfanilamide in excess of 25 mg per 100 ml, use 5 ml filtrate and 5 ml 2.4% trichloracetic acid (2) in Step 6 and multiply the values obtained from the calibration table by 2.

1. In a 125 ml Erlenmeyer flask place
    41 ml distilled water
    1 ml sample from an Ostwald-Folin pipette, thoroughly rinsing out the pipette by drawing in and expelling some of the mixture several times.
2. Mix by rotatory motion and allow to stand for 10 minutes.
3. Add 8 ml 15% trichloracetic acid (1), drop by drop, while constantly agitating the flask.
4. Stopper with a clean rubber stopper, shake vigorously for 30 seconds and allow to stand for 5 minutes.
5. Filter through a dry 9 cm Whatman #12 paper into a dry test tube, refiltering the first few drops, if necessary, to obtain a crystal-clear filtrate—RESERVE PORTION FOR TOTAL SULFANILAMIDE ANALYSIS (Method S-23a).
6. In a 25 ml graduated test tube (sample) place
    10 ml filtrate.
7. In another 25 ml graduated test tube (reagent blank) place
    10 ml 2.4% trichloracetic acid (2).
8. To each tube add 0.5 ml freshly prepared 0.1% sodium nitrite (3).

9. Mix and allow to stand away from direct sunlight for 10 minutes.
10. Add 0.5 ml 0.5% ammonium sulfamate (4).
11. Mix and allow to stand for 2 minutes.
12. Add 0.5 ml 0.1% N-(1-naphthyl)-ethylenediamine dihydrochloride (5).
13. Mix and allow to stand away from direct light for 2 minutes.
14. Dilute to 25 ml with distilled water and mix by inversion—transfer to cuvettes and make certain that the solutions are free of gas bubbles.
15. Measure the transmittancy of the sample against the reagent blank set at 100% transmission at a wave length of **545** mμ and obtain the concentration of free sulfanilamide from the calibration table.

CALIBRATION PROCEDURE

Special Apparatus

Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.

Reagents

1. **Trichloracetic Acid, 15% Aqueous.** Same as for Test Procedure.
3. **Sodium Nitrite, 0.1% Aqueous.** Same as for Test Procedure.
4. **Ammonium Sulfamate, 0.5% Aqueous.** Same as for Test Procedure.
5. **N-(1-Naphthyl)-Ethylenediamine Dihydrochloride, 0.1% Aqueous (Bratton-Marshall).** Same as for Test Procedure.
6. **Sulfanilamide Standards**
    (a) Sulfanilamide stock standard (1 ml contains 0.1 mg sulfanilamide). In a 100 ml volumetric flask place 10 mg sulfanilamide ($C_6H_8N_2O_2S$—U.S.P. Reference Standard); add approximately 80 ml distilled water, previously heated to 50° C. and shake until dissolved; allow to cool and dilute to volume with distilled water. Stable for several months in refrigerator.
    (b) Dilute standard (1 ml contains 0.025 mg sulfanilamide). In a 100 ml volumetric flask place 25 ml stock standard (6a) and dilute to volume with distilled water. Prepare just before use.
    (c) Calibration standards. In a series of six accurately calibrated 50 ml volumetric flasks place respectively 0, 2, 4, 6, 8 and 10 ml of the dilute standard (6b); to each flask add 8 ml 15% trichloracetic acid (1), mix and dilute to volume with distilled water. These standards represent concentrations of sulfanilamide equivalent to 0, 5, 10, 15, 20 and 25 mg per 100 ml sample. Prepare just before use and proceed immediately as follows.

Technic

1. Transfer 10 ml of each calibration standard (6c) to a correspondingly marked accurately calibrated 25 ml volumetric flask.
2. Proceed exactly as outlined in the Test Procedure, Steps 8–14 inclusive.
3. Set the zero concentration standard at 100% transmission at a wave length of **545** mμ and record the transmittancy of the 5, 10, 15, 20 and 25 mg per cent standards. The transmittancy of the 25 mg standard should be approximately 22%.
4. Plot the observed values on semi-log graph paper—the points fall on a very slight curve which passes through the origin (Calibration Curve S-22).
5. Prepare a calibration table from the T-C values obtained from the plotted curve.

Method S-22b

DETERMINATION IN URINE

SAMPLE

Aliquot of a 24-hour specimen.

TEST PROCEDURE

Special Apparatus

As listed under the Test Procedure for blood.

Reagents

1. **Trichloracetic Acid, 15% Aqueous.** Same as for blood.
2. **Trichloracetic Acid, 2.4% Aqueous.** Same as for blood.
3. **Sodium Nitrite, 0.1% Aqueous.** Same as for blood.
4. **Ammonium Sulfamate, 0.5% Aqueous.** Same as for blood.
5. **N-(1-Naphthyl)-Ethylenediamine Dihydrochloride, 0.1% Aqueous (Bratton-Marshall).** Same as for blood.

Technic

Note: If total sulfanilamide (Method S-23b) is to be determined on the same sample, reserve a portion of the trichloracetic acid filtrate in Step 4.

For concentrations of free sulfanilamide in excess of 250 mg per 100 ml, dilute 5 ml urine with an equal volume of distilled water before proceeding with Step 1 and multiply the values obtained from the calibration table for blood by 20.

1. In a 125 ml Erlenmeyer flask place
   41 ml distilled water
   1 ml thoroughly mixed unfiltered urine from an Ostwald-Folin pipette, thoroughly rinsing out the pipette by drawing in and expelling some of the mixture several times and mix.
2. Add 8 ml 15% trichloracetic acid (1), drop by drop, while constantly agitating the flask.
3. Stopper with a clean rubber stopper, shake vigorously for 30 seconds and allow to stand for 5 minutes.
4. Filter through a dry 9 cm Whatman #12 paper into a dry test tube, refiltering the first few drops, if necessary, to obtain a crystal-clear filtrate—RESERVE PORTION FOR TOTAL SULFANILAMIDE ANALYSIS (Method S-23b).
5. In a 25 ml graduated test tube (sample) place
   1 ml filtrate
   9 ml 2.4% trichloracetic acid (2).
6. In another 25 ml graduated test tube (reagent blank) place
   10 ml 2.4% trichloracetic acid (2).
7. Proceed exactly as outlined in the Test Procedure for blood, Steps 8–14 inclusive.
8. Measure the transmittancy of the sample against the reagent blank set at 100% transmission at a wave length of **545** mμ and multiply the value from the calibration table for blood by 10 to obtain the concentration of free sulfanilamide in mg per 100 ml urine.

Method S-22c

DETERMINATION IN CEREBROSPINAL FLUID

The determination is carried out exactly as outlined in the Test Procedure for blood and the concentration of free sulfanilamide in the cerebrospinal fluid in mg per 100 ml is obtained directly from the calibration table for blood.

PROCEDURE FOR USE WITH LINEAR-SCALE PHOTOELECTRIC COLORIMETERS (SHEARD-SANFORD TYPE)

TEST PROCEDURE

Exactly as outlined; set the reagent blank at 100 and use a green filter.

CALIBRATION PROCEDURE

Exactly as outlined—use a green filter; plot the observed readings on semi-log graph paper—the points fall on a straight line passing through the origin.

PROCEDURE FOR USE WITH LOGARITHMIC-SCALE PHOTOELECTRIC COLORIMETERS (KLETT-SUMMERSON TYPE)

TEST PROCEDURE

Exactly as outlined; set the reagent blank at 0 and use a green filter.

CALIBRATION PROCEDURE

Exactly as outlined—use a green filter; plot the observed readings on linear graph paper—the points fall on a straight line passing through the origin.

Note: The above directions are also applicable to the determination of the free form of sulfacetimide (Method S-26), sulfadiazine (Method S-28), sulfaguanidine (Method S-30), sulfamerazine (Method S-32) and sulfapyridine (Method S-42).

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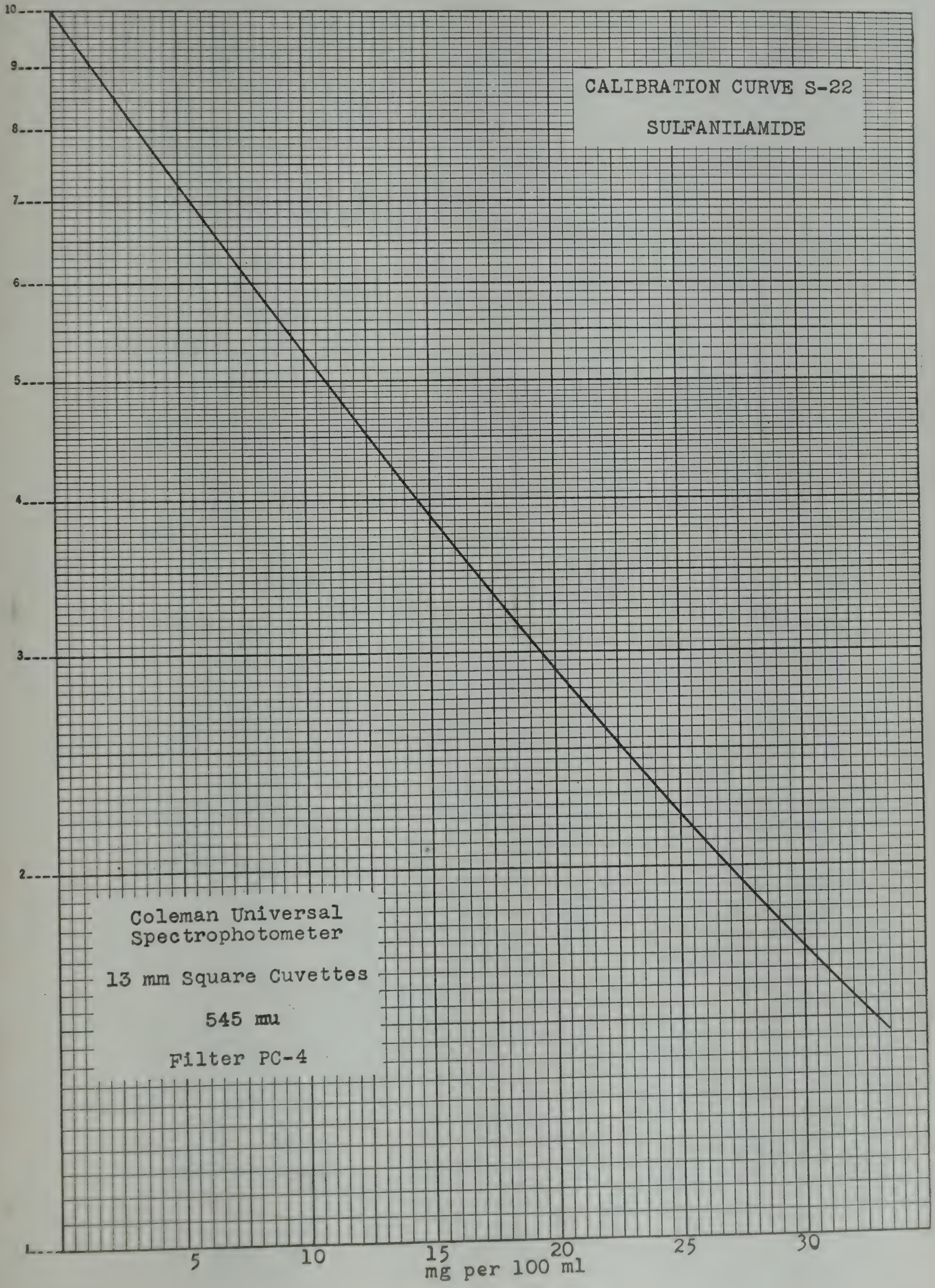
NOTES


CALIBRATION CURVE S-22
SULFANILAMIDE
Coleman Universal Spectrophotometer
13 mm Square Cuvettes
545 mu
Filter PC-4
10
9
8
7
6
5
4
3
2
1
5
10
15
20
25
30
mg per 100 ml

Meter Reading	0	1	2	3	4	5	6	7	8	9
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10										
20										
30										
40										
50										
60										
70										
80										
90										

SULFANILAMIDE, TOTAL

A spectrophotometric adaptation based on the methods and modifications of Fuller (1937), Marshall (1937), Marshall, Emerson and Cutting (1937), Hecht (1938), Marshall and Litchfield (1938), Bratton and Marshall (1939) and Holbourn and Pattle (1943)

PRINCIPLE

The aryl amino groups of the free sulfanilamide and that split off from the acetylated form by means of acid hydrolysis are determined together according to the analytical principles described for free sulfanilamide (Method S-22).

DETERMINATION IN WHOLE BLOOD, PLASMA OR SERUM

SAMPLE

See remarks under free sulfanilamide (Method S-22a). In addition to procaine and the antisyphilitic arsenicals, acetanilid and acetphenetidin produce interfering color reactions.

TEST PROCEDURE

Special Apparatus

As listed under the Test Procedure for free sulfanilamide.

Reagents

1. **Trichloracetic Acid, 15% Aqueous.** Same as for free sulfanilamide.
2. **Trichloracetic Acid, 2.4% Aqueous.** Same as for free sulfanilamide.
3. **Sodium Nitrite, 0.1% Aqueous.** Same as for free sulfanilamide.
4. **Ammonium Sulfamate, 0.5% Aqueous.** Same as for free sulfanilamide.
5. **N-(1-Naphthyl)-Ethylenediamine Dihydrochloride, 0.1% Aqueous (Bratton-Marshall).** Same as for free sulfanilamide.
7. **Hydrochloric Acid, Volumetric 4 N.** Add 400 ml concentrated hydrochloric acid (HCl—Baker & Adamson, A.C.S.) to 600 ml distilled water and mix; allow to cool; dilute 25 ml of this approximately 4 N solution to 100 ml; titrate against standard 1 N alkali using phenolphthalein as indicator; adjust accordingly and re-titrate—20 ml of a 1:4 dilution of 4 N acid require 20 ml of 1 N alkali. Preserve in a glass-stoppered bottle.

Technic

Note: If the analysis is to be carried out on an aliquot of the trichloracetic acid filtrate as reserved in the determination of free sulfanilamide (Method S-22a, Step 5), omit the following Steps 1–5 inclusive.

For concentrations of total sulfanilamide in excess of 25 mg per 100 ml, use 5 ml filtrate and 5 ml 2.4% trichloracetic acid (2) in Step 6 and multiply the values obtained from the calibration table for free sulfanilamide by 2.

1. In a 125 ml Erlenmeyer flask place
   41 ml distilled water
   1 ml sample from an Ostwald-Folin pipette, thoroughly rinsing out the pipette by drawing in and expelling some of the mixture several times.
2. Mix by rotatory motion and allow to stand for 10 minutes.
3. Add 8 ml 15% trichloracetic acid (1), drop by drop, while constantly agitating the flask.
4. Stopper with a clean rubber stopper, shake vigorously for 30 seconds and allow to stand for 5 minutes.
5. Filter through a dry 9 cm Whatman #12 paper into a dry test tube, refiltering the first few drops, if necessary, to obtain a crystal-clear filtrate.
6. In a 25 ml graduated test tube (sample) place
   10 ml filtrate.
7. In another 25 ml graduated test tube (reagent blank) place
   10 ml 2.4% trichloracetic acid (2).
8. To each tube add 0.5 ml 4 N hydrochloric acid (7) and mix.
9. Heat in actively boiling water bath for 60 minutes —do not allow fluid level to fall below 8 ml; adjust with distilled water if necessary.
10. Cool to room temperature and *adjust volume to 10 ml.*
11. To each tube add 0.5 ml freshly prepared 0.1% sodium nitrite (3).
12. Mix and allow to stand away from direct sunlight for 10 minutes.
13. Add 0.5 ml 0.5% ammonium sulfamate (4).
14. Mix and allow to stand for 2 minutes.
15. Add 0.5 ml 0.1% N-(1-naphthyl)-ethylenediamine dihydrochloride (5).
16. Mix and allow to stand away from direct light for 2 minutes.
17. Dilute to 25 ml with distilled water and mix by inversion—transfer to cuvettes and make certain that the solutions are free of gas bubbles.
18. Measure the transmittancy of the sample against the reagent blank set at 100% transmission at a wave length of 545 mμ and obtain the concentration of total sulfanilamide from the calibration table for free sulfanilamide.

CALIBRATION PROCEDURE

Values are obtained directly from the calibration table for free sulfanilamide (Method S-22).

Method S-23b

DETERMINATION IN URINE

SAMPLE

See remarks under free sulfanilamide (Method S-22b).

TEST PROCEDURE

Special Apparatus

As listed under the Test Procedure for blood.

Reagents

1. **Trichloracetic Acid, 15% Aqueous.** Same as for blood.
2. **Trichloracetic Acid, 2.4% Aqueous.** Same as for blood.
3. **Sodium Nitrite, 0.1% Aqueous.** Same as for blood.
4. **Ammonium Sulfamate, 0.5% Aqueous.** Same as for blood.
5. **N-(1-Naphthyl)-Ethylenediamine Dihydrochloride, 0.1% Aqueous (Bratton-Marshall).** Same as for blood.
7. **Hydrochloric Acid, Volumetric 4 N.** Same as for blood.

Technic

Note: If the analysis is to be carried out on an aliquot of the trichloracetic acid filtrate as reserved in the determination of free sulfanilamide (Method S-22b, Step 4), omit the following Steps 1–4 inclusive.

For concentrations of total sulfanilamide in excess of 250 mg per 100 ml, dilute 5 ml urine with an equal volume of distilled water before proceeding with Step 1 and multiply the values obtained from the calibration table for free sulfanilamide by 20.

1. In a 125 ml Erlenmeyer flask place
   41 ml distilled water
   1 ml thoroughly mixed unfiltered urine from an Ostwald-Folin pipette, thoroughly rinsing out the pipette by drawing in and expelling some of the mixture several times and mix.
2. Add 8 ml 15% trichloracetic acid (1), drop by drop, while constantly agitating the flask.
3. Stopper with a clean rubber stopper, shake vigorously for 30 seconds and allow to stand for 5 minutes.
4. Filter through a dry 9 cm Whatman #12 paper into a dry test tube, refiltering the first few drops, if necessary, to obtain a crystal-clear filtrate.
5. In a 25 ml graduated test tube (sample) place
   1 ml filtrate
   9 ml 2.4% trichloracetic acid (2).
6. In another 25 ml graduated test tube (reagent blank) place
   10 ml 2.4% trichloracetic acid (2).
7. Proceed exactly as outlined in the Test Procedure for blood, Steps 8–17 inclusive.
8. Measure the transmittancy of the sample against the reagent blank set at 100% transmission at a wave length of **545** mμ and multiply the value from the calibration table for free sulfanilamide by 10 to obtain the concentration of total sulfanilamide in mg per 100 ml urine.

Method S-23c

DETERMINATION IN CEREBROSPINAL FLUID

The determination is carried out exactly as outlined in the Test Procedure for blood and the concentration of total sulfanilamide in the cerebrospinal fluid in mg per 100 ml is obtained directly from the calibration table for free sulfanilamide.

PROCEDURE FOR USE WITH LINEAR-SCALE PHOTOELECTRIC COLORIMETERS (SHEARD-SANFORD TYPE)

TEST PROCEDURE

Exactly as outlined; set the reagent blank at 100 and use a green filter.

CALIBRATION PROCEDURE

Need not be performed; values obtained directly from calibration table for free sulfanilamide.

PROCEDURE FOR USE WITH LOGARITHMIC-SCALE PHOTOELECTRIC COLORIMETERS (KLETT-SUMMERSON TYPE)

TEST PROCEDURE

Exactly as outlined; set the reagent blank at 0 and use a green filter.

CALIBRATION PROCEDURE

Need not be performed; values obtained directly from calibration table for free sulfanilamide.

Note: The above directions are also applicable to the determination of total sulfacetimide (Method S-26), sulfadiazine (Method S-28), sulfaguanidine (Method S-30), sulfamerazine (Method S-32) and sulfapyridine (Method S-42).

BIBLIOGRAPHY

Same as listed under Free Sulfanilamide (Method S-22).

SULFANILAMIDE, ACETYLATED

The sulfanilamide in the acetylated form is the difference between the amounts of total and free sulfanilamide.

1. Determine the concentration of total sulfanilamide by Method S-23.
2. Determine the concentration of free sulfanilamide by Method S-22.
3. Calculation—

Concentration of total sulfanilamide in mg per 100 ml − Concentration of free sulfanilamide in mg per 100 ml = Concentration of acetylated sulfanilamide in mg per 100 ml.

NOTES

NOTES

SULFACETIMIDE
FREE, TOTAL AND ACETYLATED

A spectrophotometric adaptation based on the methods and modifications of Fuller (1937), Marshall (1937), Marshall, Emerson and Cutting (1937), Hecht (1938), Marshall and Litchfield (1938), Bratton and Marshall (1939), Young, Hill, Jewett and Satterthwaite (1941) and Holbourn and Pattle (1943)

Since the absorption maximum of the azo dye produced by sulfacetimide is practically the same as that produced by sulfanilamide, sulfacetimide may be determined by following the same procedures (with the exceptions noted below) outlined for sulfanilamide (Methods S-22 and Methods S-23) and by using the sulfanilamide calibration curve (Calibration Curve S-22) and table, provided that a factor, to compensate for the difference in molecular weights and final volumes, is used. However, the actual conversion factor, as found by determination, and the theoretical conversion factor (1.24), may differ slightly. This is of no clinical significance, but, for the most exacting type of work, a calibration curve and table for sulfacetimide must be constructed.

Analyses preferably made on whole blood. For concentrations of sulfacetimide in excess of 25 mg per 100 ml blood or 250 mg per 100 ml urine, see notes for sulfanilamide.

Method S-26a

FREE SULFACETIMIDE

Determine as outlined for free sulfanilamide (Methods S-22) except that the final dilution is made to 20 ml instead of 25 ml; multiply the values from the calibration table by 1.02 to obtain the concentration of free sulfacetimide in mg per 100 ml sample. If a calibration table has been prepared for sulfacetimide, the values are obtained directly.

Method S-26b

TOTAL SULFACETIMIDE

Determine as outlined for total sulfanilamide (Methods S-23) except that the final dilution is made to 20 ml instead of 25 ml; multiply the values from the calibration table by 1.02 to obtain the concentration of total sulfacetimide in mg per 100 ml sample. If a calibration table has been prepared for sulfacetimide, the values are obtained directly.

Method S-26c

ACETYLATED SULFACETIMIDE

The acetylated sulfacetimide is the difference between the total sulfacetimide and the free sulfacetimide.

CALIBRATION PROCEDURE

Note: The following calibration procedure is optional—the values in the calibration table for sulfanilamide (Method S-22), when multiplied by 1.02, are equivalent to the concentration of sulfacetimide in mg per 100 ml sample. The factor 1.02 was obtained by determination in the author's laboratory and corrects for the difference in molecular weights as well as final volumes. This factor should be determined by the analyst.

Special Apparatus

Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.

Graduated test tubes—25 ml in 0.5 ml; the 20 ml graduation must be accurately calibrated.

Reagents

1. **Trichloracetic Acid, 15% Aqueous.** Same as for sulfanilamide.
3. **Sodium Nitrite, 0.1% Aqueous.** Same as for sulfanilamide.
4. **Ammonium Sulfamate, 0.5% Aqueous.** Same as for sulfanilamide.
5. **N-(1-Naphthyl)-Ethylenediamine Dihydrochloride, 0.1% Aqueous (Bratton-Marshall).** Same as for sulfanilamide.
6. **Sulfacetimide Standards**
    (a) Sulfacetimide stock standard (1 ml contains 0.1 mg sulfacetimide). In a 100 ml volumetric flask place 10 mg sulfacetimide ($C_8H_{10}N_2O_3S$—Schering, Reagent); add 0.5 ml 1 N sodium hydroxide and 50 ml distilled water, previously heated to 50° C.

and shake until dissolved; allow to cool and dilute to volume with distilled water. Stable for several months in refrigerator.

(b) Dilute standard (1 ml contains 0.025 mg sulfacetimide). In a 100 ml volumetric flask place 25 ml stock standard (6a) and dilute to volume with distilled water. Prepare just before use.

(c) Calibration standards. In a series of six accurately calibrated 50 ml volumetric flasks place respectively 0, 2, 4, 6, 8 and 10 ml of the dilute standard (6b); to each flask add 8 ml 15% trichloracetic acid (1), mix and dilute to volume with distilled water. These standards represent concentrations of sulfacetimide equivalent to 0, 5, 10, 15, 20 and 25 mg per 100 ml sample. Prepare just before use and proceed immediately as follows.

Technic

1. Transfer 10 ml of each calibration standard (6c) to a correspondingly marked accurately calibrated 25 ml graduated test tube.
2. Proceed exactly as outlined in the Test Procedure for free sulfanilamide (Method S-22a), Steps 8–13 inclusive.
3. Dilute to 20 ml with distilled water and mix by inversion.
4. Set the zero concentration standard at 100% transmission at a wave length of 545 mμ and record the transmittancy of the 5, 10, 15, 20 and 25 mg per cent standards. The transmittancy of the 25 mg standard should be approximately 23%.
5. Plot the observed values on semi-log graph paper —the points fall on a very slight curve which passes through the origin (Calibration Curve S-26).
6. Prepare a calibration table from the T-C values obtained from the plotted curve.

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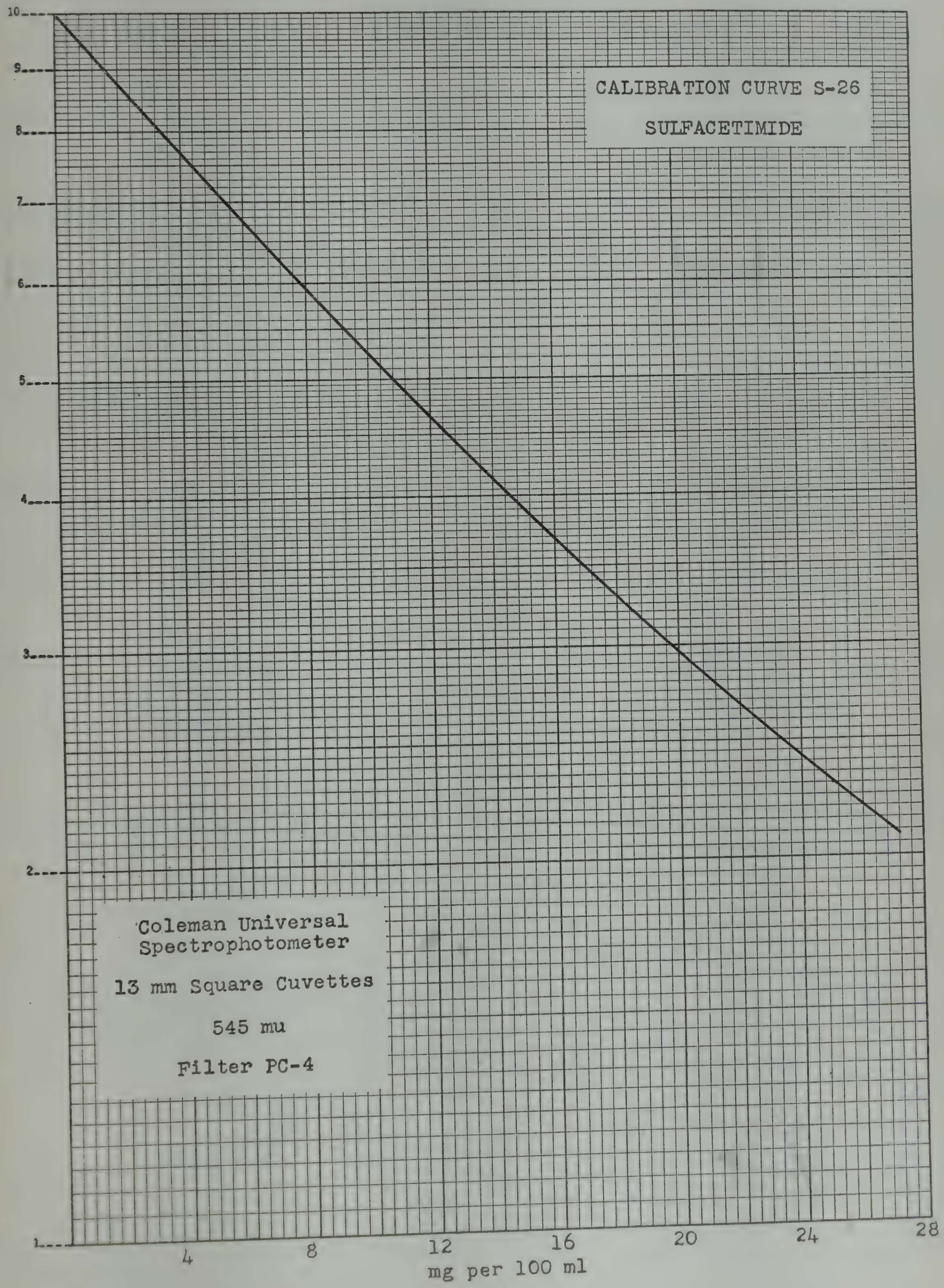
For other references see bibliography under Free Sulfanilamide (Method S-22).

NOTES


CALIBRATION CURVE S-26
SULFACETIMIDE
Coleman Universal Spectrophotometer
13 mm Square Cuvettes
545 mu
Filter PC-4
10
9
8
7
6
5
4
3
2
1
4
8
12
16
20
24
28
mg per 100 ml

Meter Reading	0	1	2	3	4	5	6	7	8	9
0										
10										
20										
30										
40										
50										
60										
70										
80										
90										

SULFADIAZINE
FREE, TOTAL AND ACETYLATED

A spectrophotometric adaptation based on the methods and modifications of Fuller (1937), Marshall (1937), Marshall, Emerson and Cutting (1937), Hecht (1938), Marshall and Litchfield (1938), Bratton and Marshall (1939), Reinhold, Flippin, Schwartz and Domm (1941) and Holbourn and Pattle (1943)

Since the absorption maximum of the azo dye produced by sulfadiazine is practically the same as that produced by sulfanilamide, sulfadiazine may be determined by following the same procedures (with the exceptions noted below) outlined for sulfanilamide (Methods S-22 and Methods S-23), and by using the sulfanilamide calibration curve (Calibration Curve S-22) and table, provided that a factor, to compensate for the difference in molecular weights and final volumes, is used. However, the actual conversion factor, as found by determination, and the theoretical conversion factor (1.45), may differ slightly. This is of no clinical significance but for the most exacting type of work, a calibration curve and table for sulfadiazine must be constructed.

Analyses preferably made on serum; whole blood contains less sulfadiazine than serum. For concentrations of sulfadiazine in excess of 25 mg per 100 ml blood or 250 mg per 100 ml urine, see notes for sulfanilamide.

Method S-28a

FREE SULFADIAZINE

Determine as outlined for free sulfanilamide (Methods S-22) except that the final dilution is made to 15 ml instead of 25 ml; multiply the values from the calibration table by 0.85 to obtain the concentration of free sulfadiazine in mg per 100 ml sample. If a calibration table has been prepared for sulfadiazine, the values are obtained directly.

Method S-28b

TOTAL SULFADIAZINE

Determine as outlined for total sulfanilamide (Methods S-23) except that the final dilution is made to 15 ml instead of 25 ml; multiply the values from the calibration table by 0.85 to obtain the concentration of total sulfadiazine in mg per 100 ml sample. If a calibration table has been prepared for sulfadiazine, the values are obtained directly.

Method S-28c

ACETYLATED SULFADIAZINE

The acetylated sulfadiazine is the difference between the total sulfadiazine and the free sulfadiazine.

CALIBRATION PROCEDURE

Note: The following calibration procedure is optional—the values in the calibration table for sulfanilamide (Method S-22), when multiplied by 0.85, are equivalent to the concentration of sulfadiazine in mg per 100 ml sample. The factor 0.85 was obtained by determination in the author's laboratory and corrects for the difference in molecular weights as well as final volumes. This factor should be determined by the analyst.

Special Apparatus

Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.

Graduated test tubes—25 ml in 0.5 ml; the 15 ml graduation must be accurately calibrated.

Reagents

1. **Trichloracetic Acid, 15% Aqueous.** Same as for sulfanilamide.
3. **Sodium Nitrite, 0.1% Aqueous.** Same as for sulfanilamide.
4. **Ammonium Sulfamate, 0.5% Aqueous.** Same as for sulfanilamide.
5. **N-(1-Naphthyl)-ethylenediamine Dihydrochloride, 0.1% Aqueous (Bratton-Marshall).** Same as for sulfanilamide.
6. **Sulfadiazine Standards**
    (a) Sulfadiazine stock standard (1 ml contains 0.1 mg sulfadiazine). In a 100 ml volumetric flask place 10 mg sulfadiazine ($C_{10}H_{10}N_4O_2S$—Lederle, Reagent); add 0.5 ml 1 N sodium hydroxide and 50 ml distilled water, previously heated to 50° C. and shake until dissolved; allow to cool and

dilute to volume with distilled water. Stable for several months in refrigerator.

(b) Dilute standard (1 ml contains 0.025 mg sulfadiazine). In a 100 ml volumetric flask place 25 ml stock standard (6a) and dilute to volume with distilled water. Prepare just before use.

(c) Calibration standards. In a series of six accurately calibrated 50 ml volumetric flasks place respectively 0, 2, 4, 6, 8 and 10 ml of the dilute standard (6b); to each flask add 8 ml 15% trichloracetic acid (1), mix and dilute to volume with distilled water. These standards represent concentrations of sulfadiazine equivalent to 0, 5, 10, 15, 20 and 25 mg per 100 ml sample. Prepare just before use and proceed immediately as follows.

Technic

1. Transfer 10 ml of each calibration standard (6c) to a correspondingly marked accurately calibrated 25 ml graduated test tube.
2. Proceed exactly as outlined in the Test Procedure for free sulfanilamide (Method S-22a), Steps 8–13 inclusive.
3. Dilute to 15 ml with distilled water and mix by inversion.
4. Set the zero concentration standard at 100% transmission at a wave length of **545** mμ and record the transmittancy of the 5, 10, 15, 20 and 25 mg per cent standards. The transmittancy of the 25 mg standard should be approximately 18%.
5. Plot the observed values on semi-log graph paper —the points fall on a very slight curve which passes through the origin (Calibration Curve S-28).
6. Prepare a calibration table from the T-C values obtained from the plotted curve.

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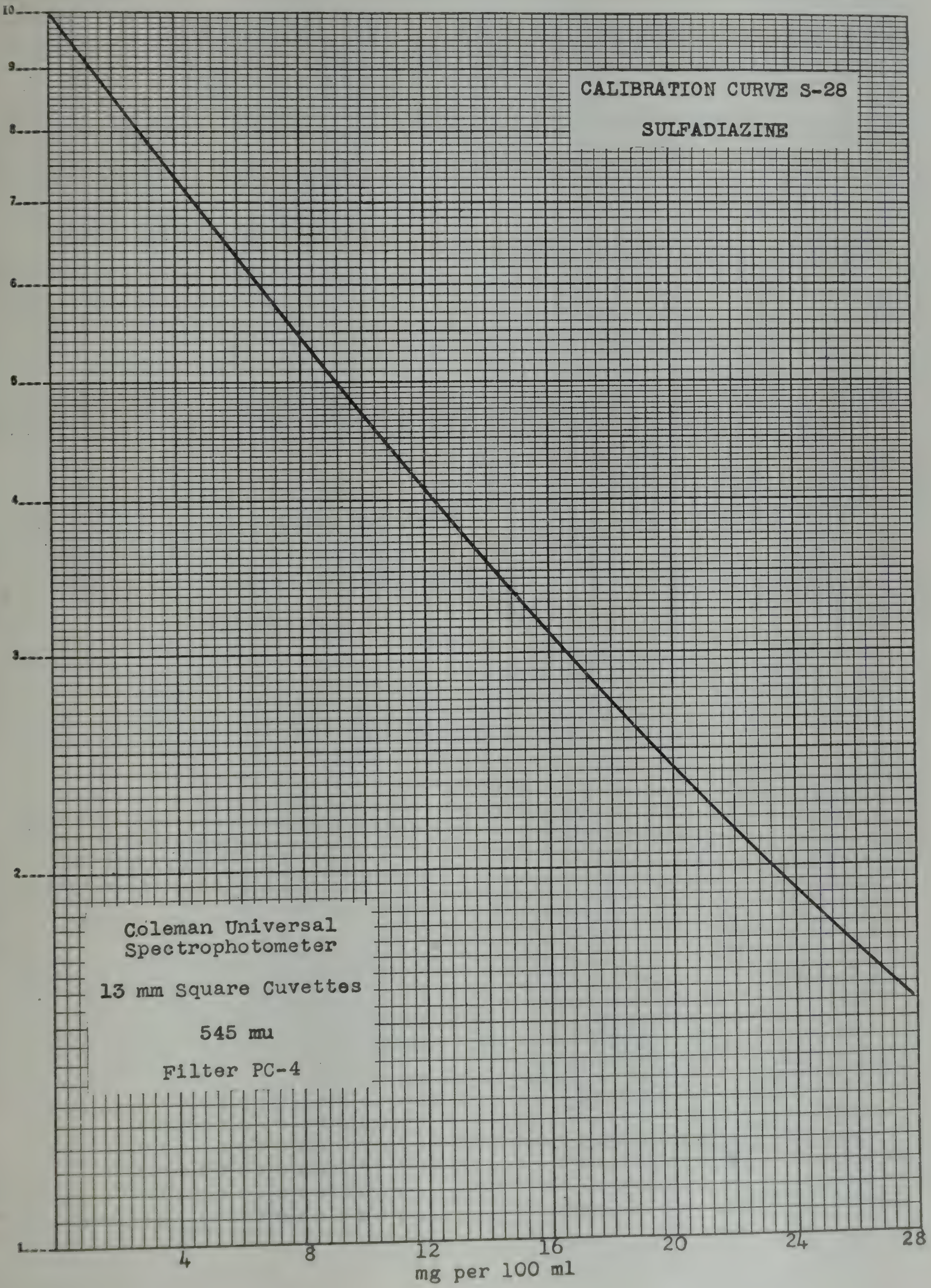
For other references see bibliography under Free Sulfanilamide (Method S-22).

NOTES


CALIBRATION CURVE S-28
SULFADIAZINE
Coleman Universal Spectrophotometer
13 mm Square Cuvettes
545 mu
Filter PC-4
10
9
8
7
6
5
4
3
2
1
4
8
12
16
20
24
28
mg per 100 ml

Meter Reading	0	1	2	3	4	5	6	7	8	9
0										
10										
20										
30										
40										
50										
60										
70										
80										
90										

SULFAGUANIDINE
FREE, TOTAL AND ACETYLATED

A spectrophotometric adaptation based on the methods and modifications of Fuller (1937), Marshall (1937), Marshall, Emerson and Cutting (1937), Hecht (1938), Marshall and Litchfield (1938), Bratton and Marshall (1939) and Holbourn and Pattle (1943)

Since the absorption maximum of the azo dye produced by sulfaguanidine is practically the same as that produced by sulfanilamide, sulfaguanidine may be determined by following the same procedures (with the exceptions noted below) outlined for sulfanilamide (Methods S-22 and Methods S-23), and by using the sulfanilamide calibration curve (Calibration Curve S-22) and table, provided that a factor, to compensate for the difference in molecular weights and final volumes, is used. However, the actual conversion factor, as found by determination, and the theoretical conversion factor (1.24), may differ slightly. This is of no clinical significance but for the most exacting type of work, a calibration curve and table for sulfaguanidine must be constructed.

Analyses preferably made on whole blood. For concentrations of sulfaguanidine in excess of 20 mg per 100 ml blood or 200 mg per 100 ml urine, see notes for sulfanilamide.

Method S-30a

FREE SULFAGUANIDINE

Determine as outlined for free sulfanilamide (Methods S-22) except that the final dilution is made to 15 ml instead of 25 ml; multiply the values from the calibration table by 0.75 to obtain the concentration of free sulfaguanidine in mg per 100 ml sample. If a calibration table has been prepared for sulfaguanidine, the values are obtained directly.

Method S-30b

TOTAL SULFAGUANIDINE

Determine as outlined for total sulfanilamide (Methods S-23) except that the final dilution is made to 15 ml instead of 25 ml; multiply the values from the calibration table by 0.75 to obtain the concentration of total sulfaguanidine in mg per 100 ml sample. If a calibration table has been prepared for sulfaguanidine, the values are obtained directly.

Method S-30c

ACETYLATED SULFAGUANIDINE

The acetylated sulfaguanidine is the difference between the total sulfaguanidine and the free sulfaguanidine.

CALIBRATION PROCEDURE

Note: The following calibration procedure is optional—the values in the calibration table for sulfanilamide (Method S-22), when multiplied by 0.75, are equivalent to the concentration of sulfaguanidine in mg per 100 ml sample. The factor 0.75 was obtained by determination in the author's laboratory and corrects for the difference in molecular weights as well as final volumes. This factor should be determined by the analyst.

Special Apparatus

Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.

Graduated test tubes—25 ml in 0.5 ml; the 15 ml graduation must be accurately calibrated.

Reagents

1. **Trichloracetic Acid, 15% Aqueous.** Same as for sulfanilamide.
3. **Sodium Nitrite, 0.1% Aqueous.** Same as for sulfanilamide.
4. **Ammonium Sulfamate, 0.5% Aqueous.** Same as for sulfanilamide.
5. **N-(1-Naphthyl)-Ethylenediamine Dihydrochloride, 0.1% Aqueous (Bratton-Marshall).** Same as for sulfanilamide.
6. **Sulfaguanidine Standards**
   (a) Sulfaguanidine stock standard (1 ml contains 0.1 mg sulfaguanidine). In a 100 ml volumetric flask place 10 mg sulfaguanidine ($C_7H_{10}N_4O_2S$—Lederle, Reagent); add approximately 80 ml distilled water, previously heated to 50° C. and shake until dissolved; allow to cool and dilute to volume

with distilled water. Stable for several months in refrigerator.

(b) Dilute standard (1 ml contains 0.025 mg sulfaguanidine). In a 100 ml volumetric flask place 25 ml stock standard (6a) and dilute to volume with distilled water. Prepare just before use.

(c) Calibration standards. In a series of five accurately calibrated 50 ml volumetric flasks place respectively 0, 2, 4, 6 and 8 ml of the dilute standard (6b); to each flask add 8 ml 15% trichloracetic acid (1), mix and dilute to volume with distilled water. These standards represent concentrations of sulfaguanidine equivalent to 0, 5, 10, 15 and 20 mg per 100 ml sample. Prepare just before use and proceed immediately as follows.

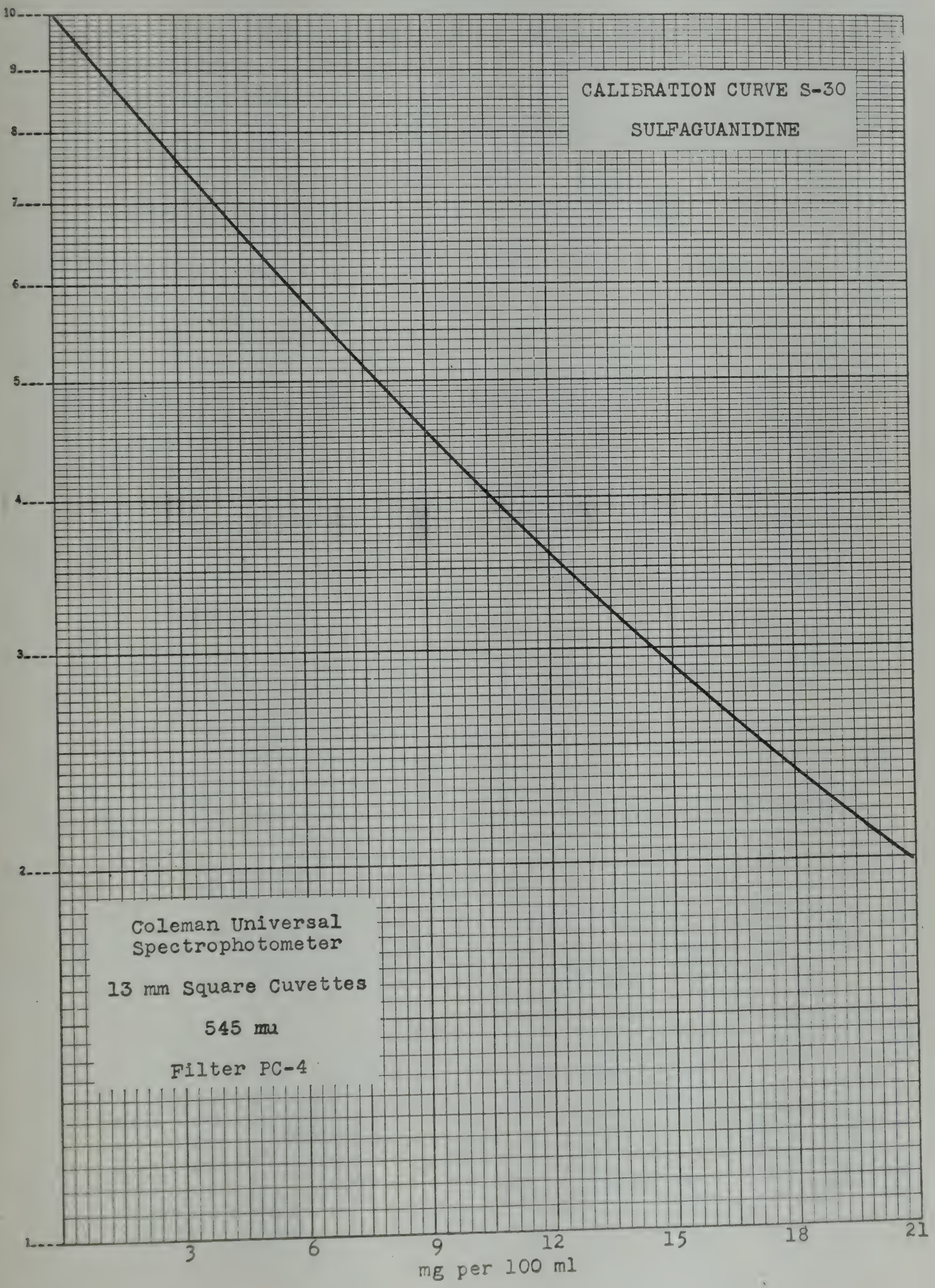
Technic

1. Transfer 10 ml of each calibration standard (6c) to a correspondingly marked accurately calibrated 25 ml graduated test tube.
2. Proceed exactly as outlined in the Test Procedure for free sulfanilamide (Method S-22a), Steps 8–13 inclusive.
3. Dilute to 15 ml with distilled water and mix by inversion.
4. Set the zero concentration standard at 100% transmission at a wave length of 545 mμ and record the transmittancy of the 5, 10, 15 and 20 mg per cent standards. The transmittancy of the 20 mg standard should be approximately 21%.
5. Plot the observed values on semi-log graph paper —the points fall on a very slight curve which passes through the origin (Calibration Curve S-30).
6. Prepare a calibration table from the T-C values obtained from the plotted curve.

BIBLIOGRAPHY

See bibliography under Free Sulfanilamide (Method S-22).

NOTES


CALIBRATION CURVE S-30
SULFAGUANIDINE
Coleman Universal Spectrophotometer
13 mm Square Cuvettes
545 mu
Filter PC-4
10
9
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15
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mg per 100 ml

Meter Reading	0	1	2	3	4	5	6	7	8	9
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SULFAMERAZINE
FREE, TOTAL AND ACETYLATED

A spectrophotometric adaptation based on the methods and modifications of Fuller (1937), Marshall (1937), Marshall, Emerson and Cutting (1937), Hecht (1938), Marshall and Litchfield (1938), Bratton and Marshall (1939), Holbourn and Pattle (1943) and Murphy, Clark and Flippin (1943)

Since the absorption maximum of the azo dye produced by sulfamerazine is practically the same as that produced by sulfanilamide, sulfamerazine may be determined by following the same procedures (with the exceptions noted below) outlined for sulfanilamide (Methods S-22 and Methods S-23), and by using the sulfanilamide calibration curve (Calibration Curve S-22) and table, provided that a factor, to compensate for the difference in molecular weights and final volumes is used. However, the actual conversion factor, as found by determination, and the theoretical conversion factor (1.53), may differ slightly. This is of no clinical significance but for the most exacting type of work, a calibration curve and table for sulfamerazine must be constructed.

Analyses preferably made on serum; whole blood contains less sulfamerazine than serum. For concentrations of sulfamerazine in excess of 25 mg per 100 ml blood or 250 mg per 100 ml urine, see notes for sulfanilamide.

Method S-32a

FREE SULFAMERAZINE

Determine as outlined for free sulfanilamide (Methods S-22) except that the final dilution is made to 15 ml instead of 25 ml; multiply the values from the calibration table by 0.9 to obtain the concentration of free sulfamerazine in mg per 100 ml sample. If a calibration table has been prepared for sulfamerazine, the values are obtained directly.

Method S-32b

TOTAL SULFAMERAZINE

Determine as outlined for total sulfanilamide (Methods S-23) except that the final dilution is made to 15 ml instead of 25 ml; multiply the values from the calibration table by 0.9 to obtain the concentration of total sulfamerazine in mg per 100 ml sample. If a calibration table has been prepared for sulfamerazine, the values are obtained directly.

Method S-32c

ACETYLATED SULFAMERAZINE

The acetylated sulfamerazine is the difference between the total sulfamerazine and the free sulfamerazine.

CALIBRATION PROCEDURE

Note: The following calibration procedure is optional—the values in the calibration table for sulfanilamide (Method S-22), when multiplied by 0.9, are equivalent to the concentration of sulfamerazine in mg per 100 ml sample. The factor 0.9 was obtained by determination in the author's laboratory and corrects for the difference in molecular weights as well as final volumes. This factor should be determined by the analyst.

Special Apparatus

Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.

Graduated test tubes—25 ml in 0.5 ml; the 15 ml graduation must be accurately calibrated.

Reagents

1. **Trichloracetic Acid, 15% Aqueous.** Same as for sulfanilamide.
3. **Sodium Nitrite, 0.1% Aqueous.** Same as for sulfanilamide.
4. **Ammonium Sulfamate, 0.5% Aqueous.** Same as for sulfanilamide.
5. **N-(1-Naphthyl)-Ethylenediamine Dihydrochloride, 0.1% Aqueous (Bratton-Marshall).** Same as for sulfanilamide.
6. **Sulfamerazine Standards**
   (a) Sulfamerazine stock standard (1 ml contains 0.1 mg sulfamerazine). In a 100 ml volumetric flask place 10 mg sulfamerazine ($C_{11}H_{12}N_4O_2S$—Sharp & Dohme, Reagent); add 0.5 ml 1 N sodium hydroxide and 50 ml distilled water, previously heated to 50° C. and shake until dissolved; allow to cool and dilute to volume with distilled water. Stable for several months in refrigerator.
   (b) Dilute standard (1 ml contains 0.025 mg sulfamerazine). In a 100 ml volumetric flask place 25 ml stock standard (6a) and dilute to volume with distilled water. Prepare just before use.

(c) Calibration standards. In a series of six accurately calibrated 50 ml volumetric flasks place respectively 0, 2, 4, 6, 8 and 10 ml of the dilute standard (6b); to each flask add 8 ml 15% trichloracetic acid (1), mix and dilute to volume with distilled water. These standards represent concentrations of sulfamerazine equivalent to 0, 5, 10, 15, 20 and 25 mg per 100 ml sample. Prepare just before use and proceed immediately as follows.

Technic

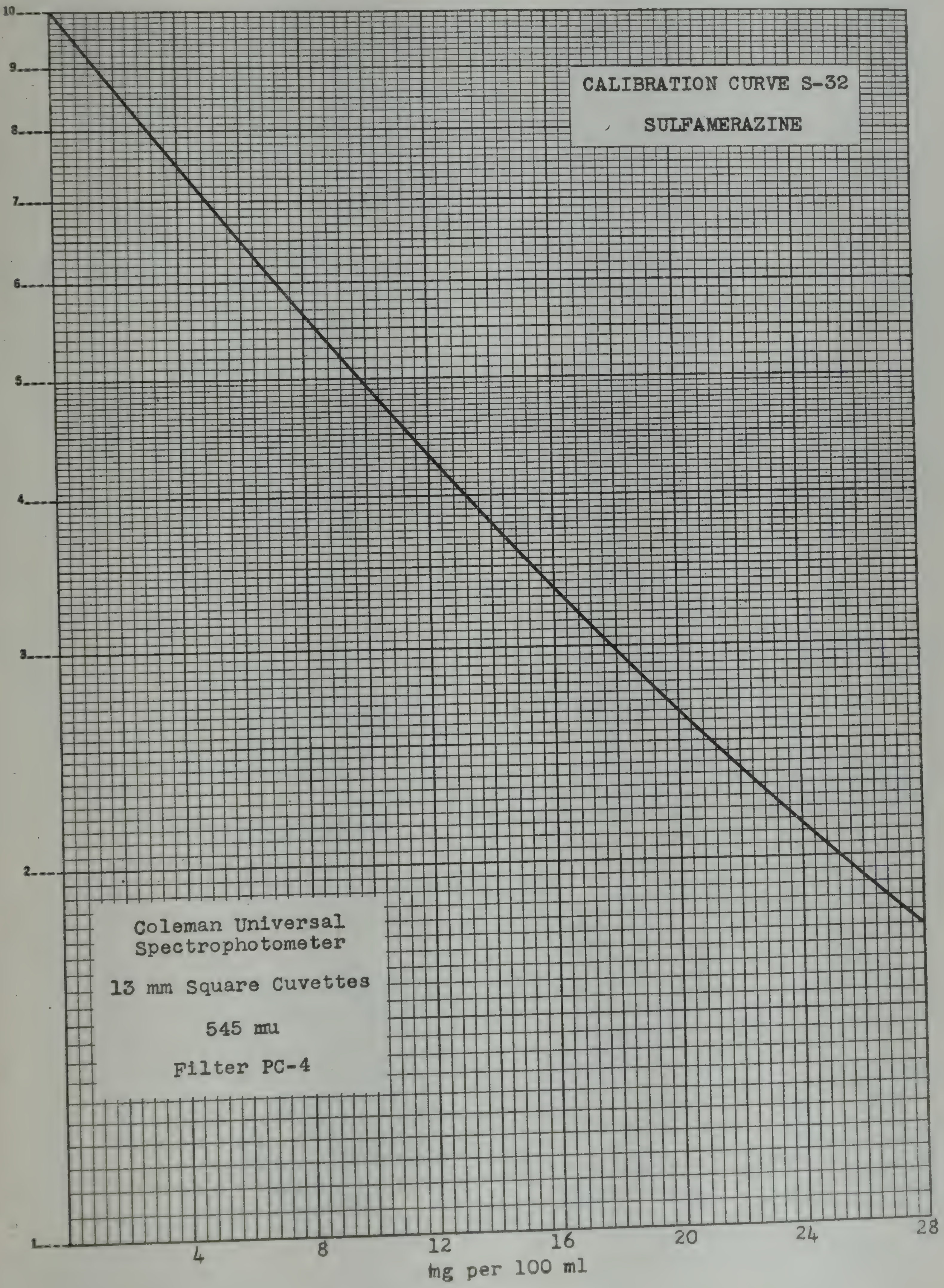
1. Transfer 10 ml of each calibration standard (6c) to a correspondingly marked accurately calibrated 25 ml graduated test tube.
2. Proceed exactly as outlined in the Test Procedure for free sulfanilamide (Method S-22a), Steps 8–13 inclusive.
3. Dilute to 15 ml with distilled water and mix by inversion.
4. Set the zero concentration standard at 100% transmission at a wave length of **545** mμ and record the transmittancy of the 5, 10, 15, 20 and 25 mg per cent standards. The transmittancy of the 25 mg standard should be approximately 20%.
5. Plot the observed values on semi-log graph paper —the points fall on a very slight curve which passes through the origin (Calibration Curve S-32).
6. Prepare a calibration table from the T-C values obtained from the plotted curve.

BIBLIOGRAPHY

Murphy, F. D., Clark, J. K., and Flippin, H. F., "Studies on 2-Sulfanilamido-4-Methyl-Pyrimidine (Sulfamerizine, Sulfamethyldiazine) in Man. I. Absorption, Distribution and Excretion," *Am. J. Med. Sc.*, **205**, 717 (1943).

For other references see bibliography under Free Sulfanilamide (Method S-22).

NOTES


CALIBRATION CURVE S-32
SULFAMERAZINE
Coleman Universal Spectrophotometer
13 mm Square Cuvettes
545 mu
Filter PC-4
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mg per 100 ml

Meter Reading	0	1	2	3	4	5	6	7	8	9
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SULFAPYRIDINE FREE, TOTAL AND ACETYLATED

A spectrophotometric adaptation based on the methods and modifications of Fuller (1937), Marshall (1937), Marshall, Emerson and Cutting (1937), Hecht (1938), Marshall and Litchfield (1938), Bratton and Marshall (1939), Scudi and Robinson (1940), James (1940), Reinhold, Flippin, Schwartz and Domm (1941) and Holbourn and Pattle (1943)

Since the absorption maximum of the azo dye produced by sulfapyridine is practically the same as that produced by sulfanilamide or sulfathiazole, sulfapyridine may be determined by following the same procedures (with the exceptions noted below) outlined for sulfanilamide (Methods S-22 and Methods S-23) or sulfathiazole (Methods S-48 and Methods S-49) and by using the appropriate calibration curve (Calibration Curve S-22 or S-48) and table, provided that appropriate factors, to compensate for the differences in molecular weights and final volumes, are used. However, the actual conversion factors, as found by determination, and the theoretical conversion factors (1.45 with sulfanilamide or 0.98 with sulfathiazole), may differ slightly. This is of no clinical significance but, for the most exacting type of work, a calibration curve and table for sulfapyridine must be constructed according to the procedure outlined for sulfathiazole (Method S-48).

Like sulfathiazole, whole blood determinations should be made on non-hemolyzed samples. Analyses preferably made on plasma; whole blood contains less sulfapyridine than plasma. For concentrations of sulfapyridine in excess of 25 mg per 100 ml blood or 250 mg per 100 ml urine, see notes for sulfanilamide or sulfathiazole, according to the technic followed.

Method S-42a

FREE SULFAPYRIDINE

Determine

(a) As outlined for free sulfanilamide (Methods S-22) except that the final dilution is made to 15 ml instead of 25 ml; multiply the values from the calibration table by 0.87 to obtain the concentration of free sulfapyridine in mg per 100 ml sample, or,

(b) Exactly as outlined for free sulfathiazole (Methods S-48) and multiply the values from the calibration table by 0.98 to obtain the concentration of free sulfapyridine in mg per 100 ml sample, or,

(c) If a calibration table has been prepared for sulfapyridine, the values are obtained directly.

Method S-42b

TOTAL SULFAPYRIDINE

Blood—Determine

(a) As outlined for total sulfanilamide (Method S-23a) except that 1 ml 4 N hydrochloric acid is used in Step 8, hydrolysis is allowed to proceed for 70 minutes and the final dilution is made to 15 ml instead of 25 ml; multiply the values from the calibration table by 0.87 to obtain the concentration of total sulfapyridine in mg per 100 ml sample, or,

(b) Exactly as outlined for total sulfathiazole (Method S-49a) and multiply the values from the calibration table by 0.98 to obtain the concentration of total sulfapyridine in mg per 100 ml sample, or,

(c) If a calibration table has been prepared for sulfapyridine, the values are obtained directly.

Urine—Determine

(a) As outlined for total sulfanilamide (Method S-23b) except that 1 ml 4 N hydrochloric acid is used in Step 7, hydrolysis is allowed to proceed for 70 minutes and the final dilution is made to 15 ml instead of 25 ml; multiply the values from the calibration table by 0.87 to obtain the concentration of total sulfapyridine in mg per 100 ml sample, or,

(b) Exactly as outlined for total sulfathiazole (Method S-49b) and multiply the values from the calibration table by 0.98 to obtain the concentration of total sulfapyridine in mg per 100 ml sample, or,

(c) If a calibration table has been prepared for sulfapyridine, the values are obtained directly.

Method S-42c

ACETYLATED SULFAPYRIDINE

The acetylated sulfapyridine is the difference between the total sulfapyridine and the free sulfapyridine.

CALIBRATION PROCEDURE

Note: The following calibration procedure is optional—the values in the calibration table for free sulfanilamide (Method S-22), when multiplied by 0.87, or, the values in the calibration table for free sulfathiazole (Method S-48), when multiplied by 0.98, are equivalent to the concentration of sulfapyridine in mg per 100 ml sample. The factors 0.87 and 0.98 were obtained by determination in the author's laboratory and correct for the differences in molecular weights as well as final volumes. These factors should be determined by the analyst.

Special Apparatus

As listed under the Calibration Procedure for sulfathiazole (Method S-48a).

Reagents

1. **Sodium Chloride, 0.85% Aqueous.** Same as for sulfathiazole.
2. **Trichloracetic Acid, 15% Aqueous.** Same as for sulfathiazole.
4. **Sodium Nitrite, 0.1% Aqueous.** Same as for sulfathiazole.
5. **Ammonium Sulfamate, 0.5% Aqueous.** Same as for sulfathiazole.
6. **N-(1-Naphthyl)-Ethylenediamine Dihydrochloride, 0.1% Aqueous (Bratton-Marshall).** Same as for sulfathiazole.
7. **Sulfapyridine Standards**
   - (a) Sulfapyridine stock standard (1 ml contains 0.1 mg sulfapyridine). In a 100 ml volumetric flask place 10 mg sulfapyridine ($C_{11}H_{11}N_3O_2S$—Merck, Reagent); add 0.5 ml 1 N sodium hydroxide and 50 ml distilled water, previously heated to 50° C. and shake until dissolved; allow to cool and dilute to volume with distilled water. Stable for several months in refrigerator.
   - (b) Dilute standard (1 ml contains 0.025 mg sulfapyridine). In a 100 ml volumetric flask place 25 ml stock standard (7a) and dilute to volume with 0.85% sodium chloride (1). Prepare just before use.
   - (c) Calibration standards. In a series of six accurately calibrated 50 ml volumetric flasks place respectively 0, 2, 4, 6, 8 and 10 ml of the dilute standard (7b); add 39, 37, 35, 33, 31 and 29 ml 0.85% sodium chloride (1) respectively; to each flask add 10 ml 15% trichloracetic acid (2) and adjust to volume with distilled water. These standards represent concentrations of sulfapyridine equivalent to 0, 5, 10, 15, 20 and 25 mg per 100 ml sample. Prepare just before use and proceed immediately as follows.

Technic

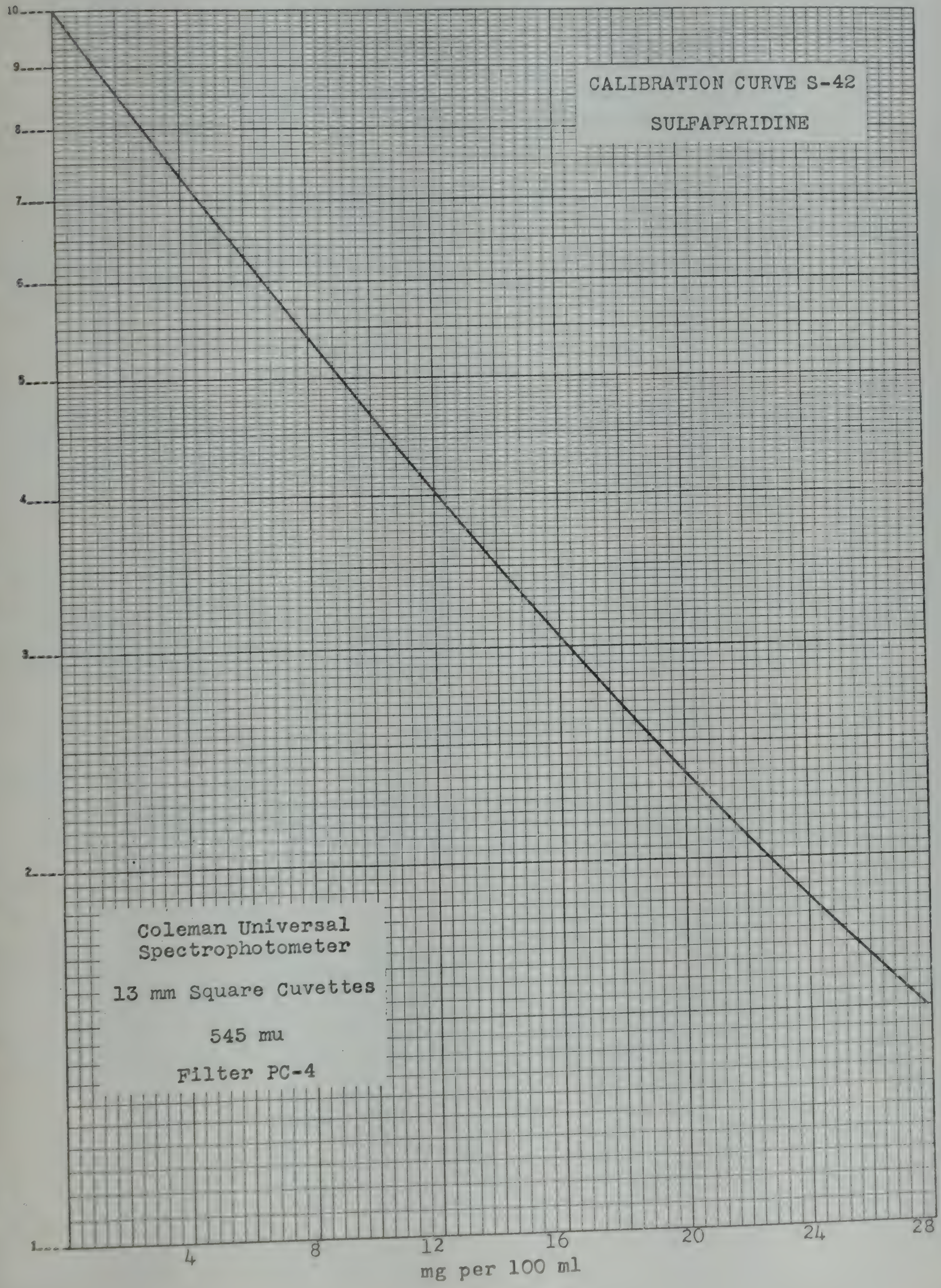
1. Transfer 10 ml of each calibration standard (7c) to a correspondingly marked accurately calibrated 25 ml graduated test tube.
2. Proceed exactly as outlined in the Test Procedure for free sulfathiazole (Method S-48a), Steps 7–13 inclusive.
3. Set the zero concentration standard at 100% transmission at a wave length of 545 mμ and record the transmittancy of the 5, 10, 15, 20 and 25 mg per cent standards. The transmittancy of the 25 mg standard should be approximately 17.5%.
4. Plot the observed values on semi-log graph paper —the points fall on a very slight curve which passes through the origin (Calibration Curve S-42).
5. Prepare a calibration table from the T-C values obtained from the plotted curve.

BIBLIOGRAPHY

James, G. V., "The Determination of Members of the Sulfanilamide Group of Drugs," *Analyst*, **65**, 206 (1940).

Scudi, J. V., and Robinson, H. J., "The Determination of Acetylsulfapyridine. II," *J. Lab. Clin. Med.*, **25**, 409 (1940).

For other references see bibliography under Free Sulfanilamide (Method S-22).


CALIBRATION CURVE S-42
SULFAPYRIDINE
Coleman Universal Spectrophotometer
13 mm Square Cuvettes
545 mu
Filter PC-4
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mg per 100 ml

Meter Reading	0	1	2	3	4	5	6	7	8	9
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SULFASUXIDINE "FREE, TOTAL AND ACETYLATED"

A spectrophotometric adaptation based on the methods and modifications of Fuller (1937), Marshall (1937), Marshall, Emerson and Cutting (1937), Hecht (1938), Marshall and Litchfield (1938), Bratton and Marshall (1939), Cooper, Gross and Hagan (1942), Welch, Mattis and Latven (1942) and Holbourn and Pattle (1943)

Sulfasuxidine (succinyl sulfathiazole) is already conjugated and cannot be acetylated. When administered, part is converted by the tissues into free sulfathiazole and part remains unconverted. Sulfasuxidine is determined by following the same procedures (with the exceptions noted below) outlined for sulfathiazole (Methods S-48 and Methods S-49) and by using the sulfathiazole calibration curve (Calibration Curve S-48) and table, provided that a factor, to compensate for the difference in molecular weights, is used. Quantitative results will not be obtained if the calibration curve and table for sulfanilamide is used.

Method S-48

FREE SULFATHIAZOLE

Determine exactly as outlined for free sulfathiazole (Methods S-48) and obtain the concentration of free sulfathiazole in mg per 100 ml sample directly from the calibration table. The concentration is not expressed in terms of sulfasuxidine.

Method S-44a

"TOTAL SULFASUXIDINE"

Determine as outlined for total sulfathiazole (Methods S-49) except that acid hydrolysis is allowed to proceed for 2 hours in Step 8; obtain the concentration of "total sulfasuxidine" (free sulfathiazole plus unhydrolyzed sulfasuxidine plus any other derivative in which substitution of the p-amino group has occurred) in terms of sulfathiazole in mg per 100 ml sample directly from the calibration table. To express in terms of sulfasuxidine multiply the concentration by 1.39.

Method S-44b

"COMBINED SULFASUXIDINE"

The concentration of "combined sulfasuxidine" is obtained as follows:

("Total sulfasuxidine" in terms of sulfathiazole in mg per 100 ml − Free sulfathiazole in mg per 100 ml) × 1.39 = Concentration of "combined sulfasuxidine" expressed in terms of sulfasuxidine in mg per 100 ml sample

CALIBRATION PROCEDURE

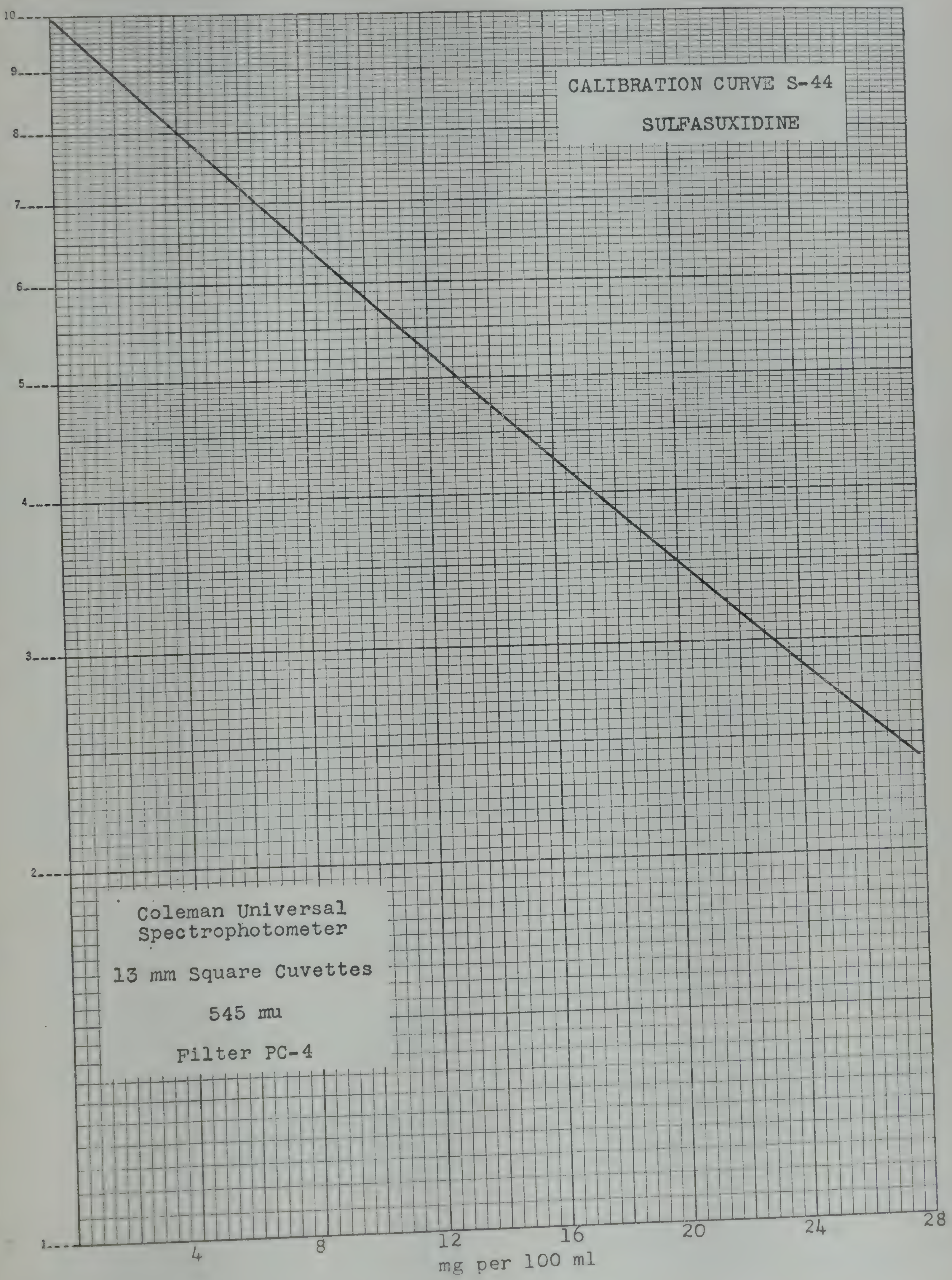
Sulfasuxidine standards are not used to prepare a calibration curve. The values in the table for sulfathiazole (Method S-48), when multiplied by 1.39 are equivalent to the concentration of sulfasuxidine in mg per 100 ml sample. When numerous sulfasuxidine determinations are required, it is convenient to prepare a calibration curve (Calibration Curve S-44) and table by calculation from the sulfathiazole curve.

BIBLIOGRAPHY

Welch, A. S., Mattis, P. A., and Latven, A. R., "A Toxicological Study of Succinyl Sulfathiazole," *J. Pharmacol. & Exper. Therap.*, 75, 231 (1942).

For other references see bibliography under Free Sulfathiazole (Method S-48).

NOTES


CALIBRATION CURVE S-44
SULFASUXIDINE
Coleman Universal Spectrophotometer
13 mm Square Cuvettes
545 mu
Filter PC-4
10
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mg per 100 ml

Meter Reading	0	1	2	3	4	5	6	7	8	9
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SULFATHIAZOLE, FREE

A spectrophotometric adaptation based on the methods and modifications of Fuller (1937), Marshall (1937), Marshall, Emerson and Cutting (1937), Hecht (1938), Marshall and Litchfield (1938), Bratton and Marshall (1939), Cooper, Gross and Hagan (1942) and Holbourn and Pattle (1943)

PRINCIPLE

The free primary aryl amino group of sulfathiazole in a protein-free trichloracetic acid filtrate of non-hemolyzed whole blood, plasma or serum is diazotized with nitrous acid, the excess nitrite being destroyed by the addition of ammonium sulfamate. The diazotized sulfonamide is coupled with N-(1-naphthyl)-ethylenediamine dihydrochloride to form a purplish-red azo dye (Bratton-Marshall reaction).

DETERMINATION IN WHOLE BLOOD, PLASMA OR SERUM

SAMPLE

Blood withdrawn at any time of the day. Anticoagulant—potassium oxalate; an excess must be avoided. Unlike sulfanilamide and certain other sulfonamides, when whole blood is used, the determination is made on a non-hemolyzed sample. Analyses preferably made on plasma; whole blood contains less sulfathiazole than plasma.

TEST PROCEDURE

Special Apparatus

Ostwald-Folin pipettes accurately calibrated "to contain" 1 ml.

Graduated test tubes—25 ml in 0.5 ml; the 15 ml graduation must be accurately calibrated.

Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.

Reagents

1. **Sodium Chloride, 0.85% Aqueous.** Dissolve 8.5 gm sodium chloride (NaCl—Merck, Special for Biological Work) in distilled water and dilute to 1000 ml.
2. **Trichloracetic Acid, 15% Aqueous.** Dilute 15 ml concentrated trichloracetic acid (a) to 100 ml with distilled water. Preserve in glass-stoppered bottle. Prepare fresh monthly.
   (a) Trichloracetic acid, concentrated; dissolve 100 gm crystalline trichloracetic acid (CCl_3-COOH—J. T. Baker, CP Analyzed) in distilled water and dilute to 100 ml. Preserve in Pyrex glass-stoppered bottle. Stability indefinite.
3. **Trichloracetic Acid, 3% Aqueous.** Dilute 20 ml 15% trichloracetic acid (1) to 100 ml with distilled water. Preserve in refrigerator. Prepare fresh weekly.
4. **Sodium Nitrite, 0.1% Aqueous.** Dilute 1 ml 10% sodium nitrite (a) to 100 ml with distilled water. Prepare fresh daily and preserve in a brown glass bottle.
   (a) Sodium nitrite, 10% aqueous; dissolve 10 gm granular sodium nitrite ($NaNO_2$—Mallinckrodt, A.C.S.) in distilled water and dilute to 100 ml. Preserve in refrigerator.
5. **Ammonium Sulfamate, 0.5% Aqueous.** Dissolve 0.5 gm crystalline ammonium sulfamate ($NH_2SO_3NH_4$—Reagent Chemicals, Special) in distilled water and dilute to 100 ml.
6. **N-(1-Naphthyl)-Ethylenediamine Dihydrochloride, 0.1% Aqueous (Bratton-Marshall).** Dissolve 100 mg N-(1-naphthyl)-ethylenediamine dihydrochloride ($C_{10}H_7HNCH_2CH_2NH_2 \cdot 2HCl$—Eastman #4835) in 100 ml distilled water. Preserve in a brown bottle in refrigerator. Stability about one month. Discard when a brownish color appears.

Technic

Note: If total sulfathiazole (Method S-49a) is to be determined on the same sample, reserve a portion of the trichloracetic acid filtrate in Step 4.

For concentrations of free sulfathiazole in excess of 25 mg per 100 ml, use 5 ml filtrate and 5 ml 3% trichloracetic acid (3) in Step 5 and multiply the values obtained from the calibration table by 2.

1. In a 125 ml Erlenmeyer flask place
   39 ml 0.85% sodium chloride (1)
   1 ml sample from an Ostwald-Folin pipette, thoroughly rinsing out the pipette by drawing in and expelling some of the mixture several times.
2. Mix gently and add 10 ml 15% trichloracetic acid (2), drop by drop, while constantly but gently agitating the flask.
3. Stopper with a clean rubber stopper, shake vigorously for 30 seconds and allow to stand for 5 minutes.
4. Filter through a dry 9 cm Whatman #12 paper into a dry test tube, re-filtering the first few drops, if necessary, to obtain a crystal-clear filtrate—RESERVE PORTION FOR TOTAL SULFATHIAZOLE ANALYSIS (Method S-49a).
5. In a 25 ml graduated test tube (sample) place
   10 ml filtrate.
6. In another 25 ml graduated test tube (reagent blank) place
   10 ml 3% trichloracetic acid (3).
7. To each tube add 0.5 ml freshly prepared 0.1% sodium nitrite (4).

8. Mix and allow to stand away from direct sunlight for 10 minutes.
9. Add 0.5 ml 0.5% ammonium sulfamate (5).
10. Mix and allow to stand for 2 minutes.
11. Add 0.5 ml 0.1% N-(1-naphthyl)-ethylenediamine dihydrochloride (6).
12. Mix and allow to stand away from direct light for 15 minutes.
13. Dilute to 15 ml with distilled water and mix by inversion—transfer to cuvettes and make certain that the solutions are free of gas bubbles.
14. Measure the transmittancy of the sample against the reagent blank set at 100% transmission at a wave length of 545 mμ and obtain the concentration of free sulfathiazole from the calibration table.

CALIBRATION PROCEDURE

Special Apparatus

Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.

Graduated test tubes—25 ml in 0.5 ml; the 15 ml graduation must be accurately calibrated.

Reagents

1. **Sodium Chloride, 0.85% Aqueous.** Same as for Test Procedure.
2. **Trichloracetic Acid, 15% Aqueous.** Same as for Test Procedure.
4. **Sodium Nitrite, 0.1% Aqueous.** Same as for Test Procedure.
5. **Ammonium Sulfamate, 0.5% Aqueous.** Same as for Test Procedure.
6. **N-(1-Naphthyl)-Ethylenediamine Dihydrochloride, 0.1% Aqueous (Bratton-Marshall).** Same as for Test Procedure.
7. **Sulfathiazole Standards**
    (a) Sulfathiazole stock standard (1 ml contains 0.1 mg sulfathiazole). In a 100 ml volumetric flask place 10 mg sulfathiazole ($C_9H_9N_3O_2S_2$—Squibb, Reagent); add 0.5 ml 1 N sodium hydroxide and 50 ml distilled water, previously heated to 50° C. and shake until dissolved; allow to cool and dilute to volume with distilled water. Stable for several months in refrigerator.
    (b) Dilute standard (1 ml contains 0.025 mg sulfathiazole). In a 100 ml volumetric flask place 25 ml stock standard (7a) and dilute to volume with 0.85% sodium chloride (1). Prepare just before use.
    (c) Calibration standards. In a series of six accurately calibrated 50 ml volumetric flasks place respectively 0, 2, 4, 6, 8 and 10 ml of the dilute standard (7b); add 39, 37, 35, 33, 31 and 29 ml 0.85% sodium chloride (1) respectively; to each flask add 10 ml 15% trichloracetic acid (2) and adjust to volume with distilled water. These standards represent concentrations of sulfathiazole equivalent to 0, 5, 10, 15, 20 and 25 mg per 100 ml sample. Prepare just before use and proceed immediately as follows.

Technic

1. Transfer 10 ml of each calibration standard (7c) to a correspondingly marked accurately calibrated 25 ml graduated test tube.
2. Proceed exactly as outlined in the Test Procedure, Steps 7–13 inclusive.
3. Set the zero concentration standard at 100% transmission at a wave length of 545 mμ and record the transmittancy of the 5, 10, 15, 20 and 25 mg per cent standards. The transmittancy of the 25 mg standard should be approximately 18%.
4. Plot the observed values on semi-log graph paper—the points fall on a very slight curve which passes through the origin (Calibration Curve S-48).
5. Prepare a calibration table from the T-C values obtained from the plotted curve.

Method S-48b

DETERMINATION IN URINE

SAMPLE

Aliquot of a 24-hour specimen.

TEST PROCEDURE

Special Apparatus

As listed under the Test Procedure for blood.

Reagents

1. **Sodium Chloride, 0.85% Aqueous.** Same as for blood.
2. **Trichloracetic Acid, 15% Aqueous.** Same as for blood.
3. **Trichloracetic Acid, 3% Aqueous.** Same as for blood.
4. **Sodium Nitrite, 0.1% Aqueous.** Same as for blood.
5. **Ammonium Sulfamate, 0.5% Aqueous.** Same as for blood.
6. **N-(1-Naphthyl)-Ethylenediamine Dihydrochloride, 0.1% Aqueous (Bratton-Marshall).** Same as for blood.

Technic

Note: If total sulfathiazole (Method S-49b) is to be determined on the same sample, reserve a portion of the trichloracetic acid filtrate in Step 4.

For concentrations of free sulfathiazole in excess of 250 mg per 100 ml, dilute 5 ml urine with an equal volume of distilled water before proceeding with Step 1 and multiply the values obtained from the calibration table for blood by 20.

1. In a 125 ml Erlenmeyer flask place
   39 ml 0.85% sodium chloride (1)
   1 ml thoroughly mixed unfiltered urine from an Ostwald-Folin pipette, thoroughly rinsing out the pipette by drawing in and expelling some of the mixture several times and mix.

Note: Although there is no necessity for the use of sodium chloride in the determination of sulfathiazole in urine, it is employed in this procedure in order that concentrations may be obtained from the calibration table for blood (Method S-49a).

2. Add 10 ml 15% trichloracetic acid (2), drop by drop, while constantly agitating the flask.
3. Stopper with a clean rubber stopper, shake vigorously for 30 seconds and allow to stand for 5 minutes.
4. Filter through a dry 9 cm Whatman #12 paper into a dry test tube, re-filtering the first few drops, if necessary, to obtain a crystal-clear filtrate—RESERVE PORTION FOR TOTAL SULFATHIAZOLE ANALYSIS (Method S-49b).
5. In a 25 ml graduated test tube (sample) place
   1 ml filtrate
   9 ml 3% trichloracetic acid (3).
6. In another 25 ml graduated test tube (reagent blank) place
   10 ml 3% trichloracetic acid (3).
7. Proceed exactly as outlined in the Test Procedure for blood, Steps 7–13 inclusive.
8. Measure the transmittancy of the sample against the reagent blank set at 100% transmission at a wave length of 545 mμ and multiply the value from the calibration table for blood by 10 to obtain the concentration of free sulfathiazole in mg per 100 ml urine.

Method S-48c

DETERMINATION IN CEREBROSPINAL FLUID

The determination is carried out exactly as outlined in the Test Procedure for blood and the concentration of free sulfathiazole in the cerebrospinal fluid in mg per 100 ml is obtained directly from the calibration table for blood.

PROCEDURE FOR USE WITH LINEAR-SCALE PHOTOELECTRIC COLORIMETERS (SHEARD-SANFORD TYPE)

TEST PROCEDURE

Exactly as outlined; set the reagent blank at 100 and use a green filter.

CALIBRATION PROCEDURE

Exactly as outlined—use a green filter; plot the observed readings on semi-log graph paper—the points fall on a straight line passing through the origin.

PROCEDURE FOR USE WITH LOGARITHMIC-SCALE PHOTOELECTRIC COLORIMETERS (KLETT-SUMMERSON TYPE)

TEST PROCEDURE

Exactly as outlined; set the reagent blank at 0 and use a green filter.

CALIBRATION PROCEDURE

Exactly as outlined—use a green filter; plot the observed readings on linear graph paper—the points fall on a straight line passing through the origin.

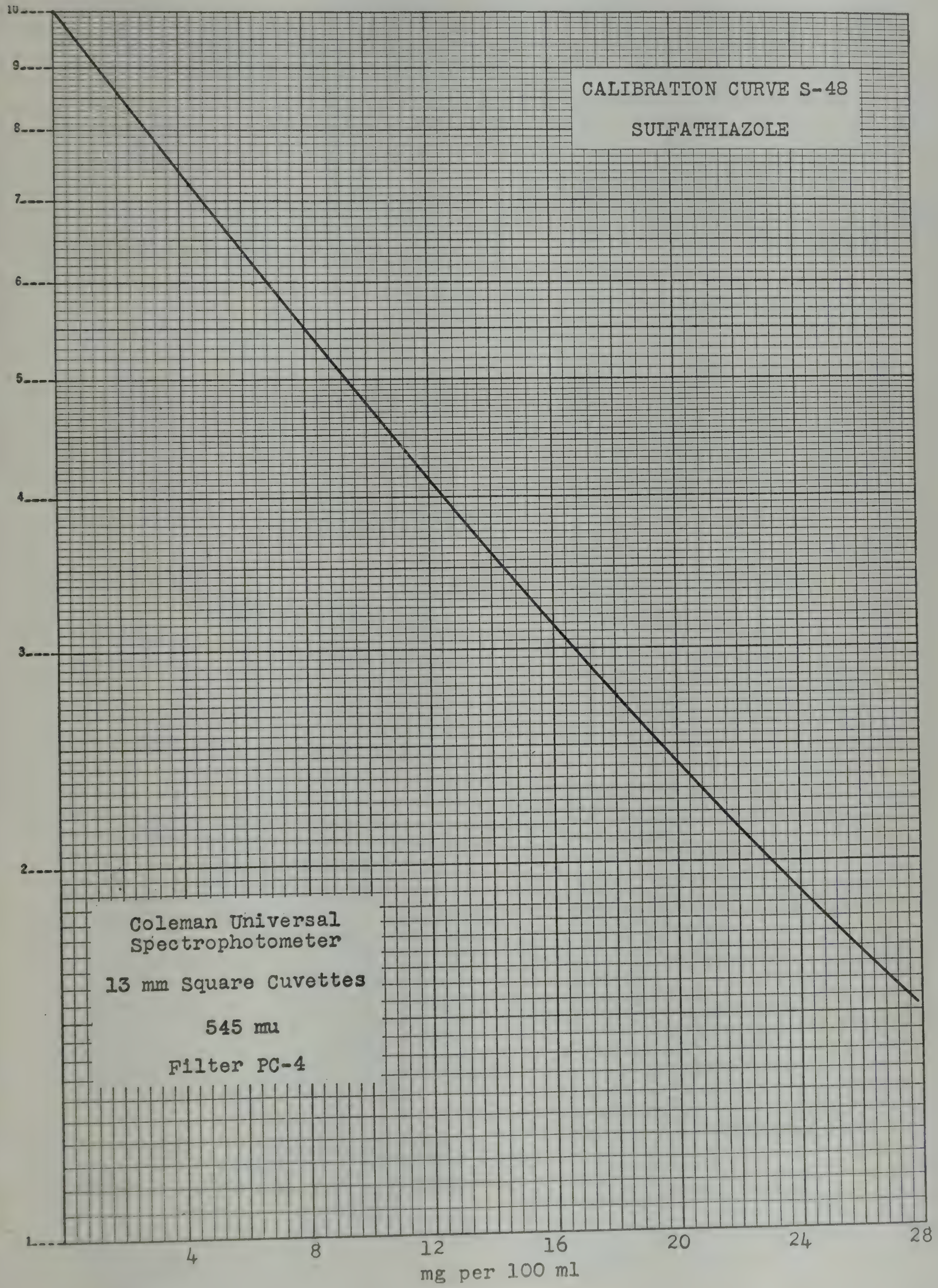
Note: The above directions are also applicable to the determination of the free form of sulfapyridine (Method S-42).

BIBLIOGRAPHY

Cooper, F. B., Gross, P., and Hagan, M. L., "Determination and Distribution of Sulfathiazole in Blood," *Am. J. Clin. Path.*, **12**, 149 (1942).

For other references see bibliography under Free Sulfanilamide (Method S-22).

NOTES


CALIBRATION CURVE S-48
SULFATHIAZOLE
Coleman Universal Spectrophotometer
13 mm Square Cuvettes
545 mu
Filter PC-4
10
9
8
7
6
5
4
3
2
1
4
8
12
16
20
24
28
mg per 100 ml

Meter Reading	0	1	2	3	4	5	6	7	8	9
0										
10										
20										
30										
40										
50										
60										
70										
80										
90										

SULFATHIAZOLE, TOTAL

A spectrophotometric adaptation based on the methods and modifications of Fuller (1937), Marshall (1937), Marshall, Emerson and Cutting (1937), Hecht (1938), Marshall and Litchfield (1938), Bratton and Marshall (1939), Cooper, Gross and Hagan (1942) and Holbourn and Pattle (1943)

PRINCIPLE

The aryl amino groups of the free sulfathiazole and that split off from the acetylated form by means of acid hydrolysis are determined together according to the analytical principles described for free sulfathiazole (Method S-48).

DETERMINATION IN WHOLE BLOOD, PLASMA OR SERUM

SAMPLE

See remarks under free sulfathiazole (Method S-48a).

TEST PROCEDURE

Special Apparatus

As listed under the Test Procedure for free sulfathiazole.

Reagents

1. **Sodium Chloride, 0.85% Aqueous.** Same as for free sulfathiazole.
2. **Trichloracetic Acid, 15% Aqueous.** Same as for free sulfathiazole.
3. **Trichloracetic Acid, 3% Aqueous.** Same as for free sulfathiazole.
4. **Sodium Nitrite, 0.1% Aqueous.** Same as for free sulfathiazole.
5. **Ammonium Sulfamate, 0.5% Aqueous.** Same as for free sulfathiazole.
6. **N-(1-Naphthyl)-Ethylenediamine Dihydrochloride, 0.1% Aqueous (Bratton-Marshall).** Same as for free sulfathiazole.
8. **Hydrochloric Acid, Volumetric 10 N.** Add 850 ml concentrated hydrochloric acid (HCl—Baker & Adamson, A.C.S.) to 150 ml distilled water and mix; allow to cool; dilute 10 ml of this approximately 10 N solution to 100 ml; titrate against standard 1 N alkali using phenolphthalein as indicator; adjust accordingly and re-titrate—20 ml of a 1:10 dilution of 10 N acid require 20 ml of 1 N alkali. Preserve in glass-stoppered bottle.

Technic

Note: If the analysis is to be carried out on an aliquot of the trichloracetic acid filtrate as reserved in the determination of free sulfathiazole (Method S-48a, Step 4), omit the following Steps 1–4, inclusive.

For concentrations of total sulfathiazole in excess of 25 mg per 100 ml, use 5 ml filtrate and 5 ml 3% trichloracetic acid (3) in Step 5 and multiply the values obtained from the calibration table for free sulfathiazole by 2.

1. In a 125 ml Erlenmeyer flask place
   39 ml 0.85% sodium chloride (1)
   1 ml sample from an Ostwald-Folin pipette, thoroughly rinsing out the pipette by drawing in and expelling some of the mixture several times.
2. Mix gently and add 10 ml 15% trichloracetic acid (2), drop by drop, while constantly but gently agitating the flask.
3. Stopper with a clean rubber stopper, shake vigorously for 30 seconds and allow to stand for 5 minutes.
4. Filter through a dry 9 cm Whatman #12 paper into a dry test tube, re-filtering the first few drops, if necessary, to obtain a crystal-clear filtrate.
5. In a 25 ml graduated test tube (sample) place
   10 ml filtrate.
6. In another 25 ml graduated test tube (reagent blank) place
   10 ml 3% trichloracetic acid (3).
7. To each tube add 1 ml 10 N hydrochloric acid (8) and mix.
8. Heat in actively boiling water bath for 75 minutes—do not allow fluid level to fall below 8 ml; adjust with distilled water if necessary.
9. Cool to room temperature and *adjust volume to 10 ml.*
10. To each tube add 0.5 ml freshly prepared 0.1% sodium nitrite (4).
11. Mix and allow to stand away from direct sunlight for 10 minutes.
12. Add 0.5 ml 0.5% ammonium sulfamate (5).
13. Mix and allow to stand for 2 minutes.
14. Add 0.5 ml 0.1% N-(1-naphthyl)-ethylenediamine dihydrochloride (6).
15. Mix and allow to stand away from direct light for 15 minutes.
16. Dilute to 15 ml with distilled water and mix by inversion—transfer to cuvettes and make certain that the solutions are free of gas bubbles.
17. Measure the transmittancy of the sample against the reagent blank set at 100% transmission at a wave length of **545** mμ and obtain the concentration of total sulfathiazole from the calibration table for free sulfathiazole.

CALIBRATION PROCEDURE

Values are read directly from the calibration table for free sulfathiazole (Method S-48).

Method S-49b

DETERMINATION IN URINE

SAMPLE

See remarks under free sulfathiazole (Method S-48b).

TEST PROCEDURE

Special Apparatus

As listed under the Test Procedure for blood.

Reagents

1. **Sodium Chloride, 0.85% Aqueous.** Same as for blood.
2. **Trichloracetic Acid, 15% Aqueous.** Same as for blood.
3. **Trichloracetic Acid, 3% Aqueous.** Same as for blood.
4. **Sodium Nitrite, 0.1% Aqueous.** Same as for blood.
5. **Ammonium Sulfamate, 0.5% Aqueous.** Same as for blood.
6. **N-(1-Naphthyl)-Ethylenediamine Dihydrochloride, 0.1% Aqueous (Bratton-Marshall).** Same as for blood.
8. **Hydrochloric Acid, Volumetric 10 N.** Same as for blood.

Technic

Note: If the analysis is to be carried out on an aliquot of the trichloracetic acid filtrate as reserved in the determination of free sulfathiazole (Method S-48b, Step 4), omit the following Steps 1–4 inclusive.

For concentrations of total sulfathiazole in excess of 250 mg per 100 ml, dilute 5 ml urine with an equal volume of distilled water before proceeding with Step 1 and multiply the values obtained from the calibration table for free sulfathiazole by 20.

1. In a 125 ml Erlenmeyer flask place
   39 ml 0.85% sodium chloride (1)
   1 ml thoroughly mixed unfiltered urine from an Ostwald-Folin pipette, thoroughly rinsing out the pipette by drawing in and expelling some of the mixture several times and mix.

   Note: See remarks under free sulfathiazole (Method S-48b).
2. Add 10 ml 15% trichloracetic acid (2), drop by drop, while constantly agitating the flask.
3. Stopper with a clean rubber stopper, shake vigorously for 30 seconds and allow to stand for 5 minutes.
4. Filter through a dry 9 cm Whatman #12 paper into a dry test tube, re-filtering the first few drops, if necessary, to obtain a crystal-clear filtrate.
5. In a 25 ml graduated test tube (sample) place
   1 ml filtrate
   9 ml 3% trichloracetic acid (3).
6. In another 25 ml graduated test tube (reagent blank) place
   10 ml 3% trichloracetic acid (3).
7. Proceed exactly as outlined in the Test Procedure for blood, Steps 7–16 inclusive.
8. Measure the transmittancy of the sample against the reagent blank set at 100% transmission at a wave length of 545 mμ and multiply the value from the calibration table for free sulfathiazole by 10 to obtain the concentration of total sulfathiazole in mg per 100 ml urine.

Method S-49c

DETERMINATION IN CEREBROSPINAL FLUID

The determination is carried out exactly as outlined in the Test Procedure for blood and the concentration of total sulfathiazole in the cerebrospinal fluid in mg per 100 ml is obtained directly from the calibration table for free sulfathiazole.

◆

PROCEDURE FOR USE WITH LINEAR-SCALE PHOTOELECTRIC COLORIMETERS (SHEARD-SANFORD TYPE)

TEST PROCEDURE

Exactly as outlined; set the reagent blank at 100 and use a green filter.

CALIBRATION PROCEDURE

Need not be performed; values obtained directly from calibration table for free sulfathiazole.

◆

PROCEDURE FOR USE WITH LOGARITHMIC-SCALE PHOTOELECTRIC COLORIMETERS (KLETT-SUMMERSON TYPE)

TEST PROCEDURE

Exactly as outlined; set the reagent blank at 0 and use a green filter.

CALIBRATION PROCEDURE

Need not be performed; values obtained directly from calibration table for free sulfathiazole.

Note: The above directions are also applicable to the determination of total sulfapyridine (Method S-42) and sulfasuxidine (Method S-44).

BIBLIOGRAPHY

Same as listed under Free Sulfathiazole (Method S-49).

SULFATHIAZOLE, ACETYLATED

The sulfathiazole in the acetylated form is the difference between the amounts of total and free sulfathiazole.

1. Determine the concentration of total sulfathiazole by Method S-49.
2. Determine the concentration of free sulfathiazole by Method S-48.
3. Calculation—

   Concentration of total sulfathiazole in mg per 100 ml − Concentration of free sulfathiazole in mg per 100 ml = Concentration of acetylated sulfathiazole in mg per 100 ml

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NOTES

NOTES

SULFATE, INORGANIC

A spectrophotometric adaptation based on the methods and modifications of Yoshimatsu (1926), Hubbard (1927), Kahn and Leiboff (1928), Wakefield (1929), Letonoff and Reinhold (1936), Marenzi and Banfi (1939) and Klein (1944)

PRINCIPLE

The protein-free filtrate is treated with aluminum chloride in alkaline solution to remove phosphates and other interfering substances. Upon re-acidification, inorganic sulfate is precipitated with benzidine and re-dissolved in hydrochloric acid. The sulfate is then diazotized, excess nitrite being destroyed by ammonium sulfamate and coupled with N-(1-naphthyl)-ethylenediamine dihydrochloride to produce a purple color.

DETERMINATION IN WHOLE BLOOD, PLASMA OR SERUM

SAMPLE

Blood withdrawn during post-absorptive state. Fluorides or oxalates cannot be used as anticoagulants for whole blood or plasma—the freshly drawn blood must be defibrinated by vigorous stirring with a glass rod. Heparin may be used for the inorganic sulfate determination but for no other sulfur fraction. Analyses preferably made on serum.

TEST PROCEDURE

Special Apparatus

Ostwald-Folin pipettes accurately calibrated "to deliver" 4 ml.

Centrifuge tubes, 15 ml, Pyrex, glass-stoppered, with sharply pointed tips.

Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.

Note: Glassware must be absolutely free of sulfates. Dichromate-sulfuric acid solution should not be used for cleaning—instead, use a mixture of 300 ml concentrated nitric acid and 1000 ml 5% potassium dichromate; rinse thoroughly with distilled water and dry before use.

Reagents

Note: The distilled water and reagents must be sulfate-free and should show no discernible precipitate with barium chloride.

1. **Water, Distilled, Sulfate-Free.** Redistil distilled water from an all-glass Pyrex distillation apparatus and test for the presence of sulfate with barium chloride. This must be used for the preparation of all reagents and standards and where indicated in the test procedure.
2. **Trichloracetic Acid, 10% Aqueous.** Dilute 10 ml concentrated trichloracetic acid (a) to 100 ml with sulfate-free distilled water (1). Preserve in Pyrex glass-stoppered bottle. Prepare fresh weekly.
   (a) Trichloracetic acid, concentrated; dissolve 100 gm crystalline trichloracetic acid (CCl_3-COOH—Baker & Adamson, Reagent) in sulfate-free distilled water (1) and dilute to 100 ml. Preserve in Pyrex glass-stoppered bottle. Stability indefinite. Note: Trichloracetic acid, even of the grade specified, always contains traces of sulfate. It must be purified by adding 5 ml 0.5% benzidine to the molten acid and re-distilling in vacuo at a pressure of 15–20 mm.
3. **Aluminum Chloride, 1.38% Aqueous.** Dissolve 1.38 gm anhydrous aluminum chloride ($AlCl_3$—Mallinckrodt, AR) in sulfate-free distilled water (1) and dilute to 100 ml. Note: In place of anhydrous aluminum chloride, 2.5 gm of the crystalline form may be dissolved in 100 ml water —the anhydrous form is preferred because of its much lower sulfate content.
4. **Ammonia Solution.** Dilute 20 ml ammonium hydroxide (NH_4OH—Baker & Adamson, A.C.S.) to 100 ml with sulfate-free distilled water (1) and add 5 ml 0.02% methyl red indicator solution (a).
   (a) Methyl red indicator solution, 0.02% aqueous; dissolve 10.8 mg of the sodium salt of methyl red ($C_{15}H_{14}O_2N_3Na$—Coleman & Bell, Water soluble) in sulfate-free distilled water (1) and dilute to 50 ml.
5. **Trichloracetic Acid, 5% in Acetone (Marenzi-Banfi).** Add 5 gm crystalline re-distilled trichloracetic acid (see note under Reagent #2a) to 100 ml re-distilled anhydrous acetone ($(CH_3)_2CO$ —Baker & Adamson, A.C.S.) and shake until dissolved. Preserve in Pyrex glass-stoppered bottle. Prepare fresh daily. Solutions showing a yellow tinge should be discarded.
6. **Benzidine, 1.5% in Acetone (Marenzi-Banfi).** Add 1.5 gm benzidine ($(C_6H_4)_2(NH_2)_2$—Matheson) to 100 ml re-distilled anhydrous acetone ($(CH_3)_2$-CO—Baker & Adamson, A.C.S.) and shake until dissolved; filter, if necessary, through a Whatman #40 paper. Prepare fresh daily.
7. **Hydrochloric Acid, Volumetric 0.2 N.** Slowly add 17 ml concentrated hydrochloric acid (HCl—Baker & Adamson, A.C.S.) to 1000 ml sulfate-free distilled water (1) and mix well; standardize against 0.2 N alkali using phenolphthalein as indicator.
8. **Sodium Nitrite, 0.1% Aqueous.** Dilute 1 ml 10% sodium nitrite (a) to 100 ml with sulfate-free distilled water (1). Prepare fresh daily and preserve in a brown glass bottle.

(a) Sodium nitrite, 10% aqueous; dissolve 10 gm granular sodium nitrite ($NaNO_2$—Mallinckrodt, A.C.S.) in sulfate-free distilled water (1) and dilute to 100 ml. Preserve in refrigerator.

9. **Ammonium Sulfamate, 0.5% Aqueous.** Dissolve 0.5 gm crystalline ammonium sulfamate ($NH_2SO_3NH_4$—Reagent Chemicals, Special) in sulfate-free distilled water (1) and dilute to 100 ml. Stable indefinitely; discard if mold-growth occurs.
10. **N-(1-Naphthyl)-Ethylenediamine Dihydrochloride, 0.1% Aqueous.** Dissolve 100 mg N-(1-naphthyl)-ethylenediamine dihydrochloride ($C_{10}H_7HNCH_2CH_2NH_2 \cdot 2HCl$—Eastman #4835) in 100 ml sulfate-free distilled water (1). Preserve in a brown glass bottle in refrigerator. Stability about 1 month. Discard when a brownish color appears.

Technic

Note: For concentrations of inorganic sulfate in excess of 7.5 mg per 100 ml, add a larger volume of 0.2 N hydrochloric acid (7) in Step 18, use an aliquot and correct the values obtained from the calibration table accordingly.

1. In a 15 ml centrifuge tube place
   8 ml 10% trichloracetic acid (2)
   4 ml sample from an Ostwald-Folin pipette, added drop by drop, while constantly agitating the tube.
2. Shake vigorously for 1 minute and allow to stand for 20 minutes.
3. Centrifuge at 1500 rpm for 5 minutes.
4. Transfer 6 ml clear centrifugate to another 15 ml centrifuge tube.
5. Add 0.5 ml 1.38% aluminum chloride (3) and mix thoroughly by rapidly twirling the tube between the hands.
6. Add 0.5 ml ammonia solution (4) and mix—distinct alkalinity is evidenced by a yellow color but an excess of ammonia must be avoided.
7. Allow to stand for 10 minutes until the aluminum hydroxide flocculates.
8. Centrifuge at 2000 rpm for 5 minutes.
9. Transfer 3.5 ml clear centrifugate to a 15 ml glass-stoppered centrifuge tube with sharply pointed tip.
10. Neutralize by the addition of freshly prepared 5% trichloracetic acid in acetone (5)—about 1 ml is required.
11. Add an additional 2 ml 5% trichloracetic acid in acetone (5)—the solution must now be distinctly acid as evidenced by a red color.
12. Add 6 ml freshly prepared 1.5% benzidine in acetone (6) and mix.
13. Stopper the tube and place in the refrigerator at 0° C. for 2 hours—longer does no harm.
14. Centrifuge the stoppered tube at 3000 rpm for 15 minutes.
15. Decant the supernatant fluid and discard; drain the tube for 3 minutes by inverting on a filter paper moistened with acetone.
16. Break up the precipitate with a thin glass rod and add 10 ml 5% trichloracetic acid in acetone (5), washing down the walls of the tube and rinsing off the rod at the same time.
17. Centrifuge and drain for 5 minutes as in Steps 14 and 15; wipe the lip of the tube dry with a tissue.
18. Add 2 ml 0.2 N hydrochloric acid (7) and place the tube in a water bath at 60° C. for 5 minutes, shaking frequently, until the precipitate is dissolved—the contents must not be allowed to boil.
19. Cool to room temperature and transfer the contents of the tube to an 18 × 150 mm test tube (sample), rinsing the centrifuge tube with three 1–2 ml portions sulfate-free distilled water (1), adding the washings to the test tube, bringing the volume to 6–8 ml.
20. In another 18 × 150 mm test tube (reagent blank) place 6–8 ml sulfate-free distilled water (1).
21. Place both tubes in a beaker half-filled with ice cold water.
22. To each tube add 1 ml 0.1% sodium nitrite (8), mix and allow to remain in the cold water for 3 minutes.
23. Add 1 ml 0.5% ammonium sulfamate (9), mix and allow to remain in the cold water for 2 minutes.
24. Remove the tubes from the bath and add 1 ml 0.1% N-(1-naphthyl)-ethylenediamine dihydrochloride (10) and mix thoroughly.
25. Allow to stand at room temperature for 20 minutes.
26. Transfer the contents of the tubes to correspondingly marked 100 ml volumetric flasks, rinsing out the tubes with several small portions sulfate-free distilled water (1) and adding the washings to the flasks.
27. Dilute to volume with sulfate-free distilled water (1) and mix thoroughly by inversion.
28. Measure the transmittancy of the sample against the reagent blank set at 100% transmission at a wave length of 575 mμ and obtain the concentration of inorganic sulfate from the calibration table.

CALIBRATION PROCEDURE

Note: Two procedures are described—(A) which affords a rapid means of accurately constructing a calibration curve and table and, (B) which is recommended as a check on the analyst's technical skill. With either procedure Calibration Curve S-60 should be obtained.

Procedure (A)

Special Apparatus

Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.

Reagents

1. **Water, Distilled, Sulfate-Free.** Same as for Test Procedure.
7. **Hydrochloric Acid, Volumetric 0.2 N.** Same as for Test Procedure.
8. **Sodium Nitrite, 0.1% Aqueous.** Same as for Test Procedure.
9. **Ammonium Sulfamate, 0.5% Aqueous.** Same as for Test Procedure.
10. **N-(1-Naphthyl)-Ethylenediamine Dihydrochloride, 0.1% Aqueous.** Same as for Test Procedure.
11. **Sulfate Standards**
    - (a) Sulfate stock standard (1 ml equivalent to 0.5 mg sulfur or 1.5 mg sulfate). In a 100 ml volumetric flask place 400.15 mg dry benzidine dihydrochloride ($(C_6H_4)_2(NH_2)_2 \cdot 2HCl$—Merck, Reagent); add approximately 80 ml 0.2 N hydrochloric acid (7); shake until dissolved and dilute to volume with same. Preserve in refrigerator. Stability indefinite.
    - (b) Dilute standard (1 ml equivalent to 0.01 mg sulfur or 0.03 mg sulfate). In a 100 ml volumetric flask place 2 ml stock standard (11a) and dilute to volume with 0.2 N hydrochloric acid (7). Preserve in refrigerator. Stable for 2–3 days.
    - (c) Calibration standards. In a series of six 18 × 150 mm test tubes place respectively 0, 0.5, 1.0, 1.5, 2.0 and 2.5 ml of the dilute standard (11b); add 2.5, 2.0, 1.5, 1.0, 0.5 and 0 ml 0.2 N hydrochloric acid (7) respectively; to each add 5 ml sulfate-free distilled water (1) and mix. These standards represent concentrations of inorganic sulfate equivalent to 0, 1.5, 3.0, 4.5, 6.0 and 7.5 mg per 100 ml sample. Prepare just before use and proceed immediately as follows.

Technic

1. Place the tubes containing the calibration standards (11c) in a beaker half-filled with ice cold water.
2. Proceed exactly as outlined in the Test Procedure, Steps 22–27 inclusive.
3. Set the zero concentration standard, which should be colorless, at 100% transmission at a wave length of 575 mμ and record the transmittancy of the 1.5, 3.0, 4.5, 6.0 and 7.5 mg per cent standards. The transmittancy of the 7.5 mg standard should be approximately 32%.
4. Plot the observed values on semi-log graph paper—the points fall on a straight line which passes through the origin (Calibration Curve S-60).
5. Prepare a calibration table from the T-C values obtained from the plotted curve.

Procedure (B)

Special Apparatus

Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.

Centrifuge tubes, 15 ml, Pyrex, glass-stoppered, with sharply pointed tips.

Reagents

1. **Water, Distilled, Sulfate-Free.** Same as for Test Procedure.
2. **Trichloracetic Acid, 10% Aqueous.** Same as for Test Procedure.
3. **Aluminum Chloride, 1.38% Aqueous.** Same as for Test Procedure.
4. **Ammonia Solution.** Same as for Test Procedure.
5. **Trichloracetic Acid, 5% in Acetone (Marenzi-Banfi).** Same as for Test Procedure.
6. **Benzidine, 1.5% in Acetone (Marenzi-Banfi).** Same as for Test Procedure.
7. **Hydrochloric Acid, Volumetric 0.2 N.** Same as for Test Procedure.
8. **Sodium Nitrite, 0.1% Aqueous.** Same as for Test Procedure.
9. **Ammonium Sulfamate, 0.5% Aqueous.** Same as for Test Procedure.
10. **N-(1-Naphthyl)-Ethylenediamine Dihydrochloride, 0.1% Aqueous.** Same as for Test Procedure.
12. **Sulfate Standards**
    - (a) Sulfate stock standard (1 ml contains 0.4 mg sulfur or 1.2 mg sulfate). Dry recrystallized potassium sulfate (K_2SO_4—Reagent Chemicals, Special) at 100° C. for one hour and cool in desiccator to constant weight; transfer 217.48 mg to a 100 ml volumetric flask; add approximately 80 ml sulfate-free distilled water (1); shake until dissolved and dilute to volume. Stability indefinite.
    - (b) Dilute standard (1 ml contains 0.04 mg sulfur or 0.12 mg sulfate). In a 100 ml volumetric flask place 10 ml stock standard (12a) and dilute to volume with sulfate-free distilled water (1). Prepare just before use.
    - (c) Calibration standards. In a series of six centrifuge tubes place respectively 0, 0.5, 1.0, 1.5, 2.0 and 2.5 ml of the dilute standard (12b); add 4.0, 3.5, 3.0, 2.5, 2.0 and 1.5 ml sulfate-free distilled water (1) respectively, bringing the volume in each tube to 4 ml. These standards represent concentrations of inorganic sulfate equivalent to 0, 1.5, 3.0, 4.5, 6.0 and 7.5 mg per 100 ml sample. Prepare just before use and proceed immediately as follows.

Technic

1. To each calibration standard (12c) add 8 ml 10% trichloracetic acid (2).
2. Proceed exactly as outlined in the Test Procedure, Steps 3–27 inclusive.
3. Set the zero concentration standard, which should be colorless, at 100% transmission at a wave length of **575 mμ** and record the transmittancy of the 1.5, 3.0, 4.5, 6.0 and 7.5 mg per cent standards. The transmittancy of the 7.5 mg standard should be approximately 32%.
4. Plot the observed values on semi-log graph paper—the points fall on a straight line which passes through the origin (Calibration Curve S-60).
5. Prepare a calibration table from the T-C values obtained from the plotted curve.

CONVERSION FACTOR

$$\frac{\text{Inorganic sulfate in mg per 100 ml}}{3} = \text{Inorganic sulfate-sulfur in mg per 100 ml}$$

Method S-60b

DETERMINATION IN URINE

PRINCIPLE

Phosphates are removed by treatment with uranium acetate and the sulfate determined as outlined for blood, the same analytical principles being involved.

SAMPLE

Aliquot of a 24-hour specimen. The greater the 24-hour volume, the larger the aliquot to be taken for analysis. For normal urines a 1:50 dilution is usually sufficient.

TEST PROCEDURE

Special Apparatus

Ostwald-Folin pipettes accurately calibrated "to deliver" 1 ml.

Centrifuge tubes, 15 ml, Pyrex, glass-stoppered, with sharply pointed tips.

Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.

Reagents

1. **Water, Distilled, Sulfate-Free.** Same as for blood.
2. **Trichloracetic Acid, 10% Aqueous.** Same as for blood.
5. **Trichloracetic Acid, 5% in Acetone (Marenzi-Banfi).** Same as for blood.
6. **Benzidine, 1.5% in Acetone (Marenzi-Banfi).** Same as for blood.
7. **Hydrochloric Acid, Volumetric 0.2 N.** Same as for blood.
8. **Sodium Nitrite, 0.1% Aqueous.** Same as for blood.
9. **Ammonium Sulfamate, 0.5% Aqueous.** Same as for blood.
10. **N-(1-Naphthyl)-Ethylenediamine Dihydrochloride, 0.1% Aqueous.** Same as for blood.
13. **Uranium Acetate, 0.4% Aqueous.** Dissolve 0.4 gm crystalline uranium acetate ($(CH_3COO)_2UO_2 \cdot 2H_2O$—J. T. Baker, CP Analyzed) in sulfate-free distilled water (1) and dilute to 100 ml.

Technic

Note: For concentrations of inorganic sulfate in excess of 375 mg per 100 ml, dilute 1 ml urine to 100 ml or even more with sulfate-free distilled water (1) in Step 1 and correct the values obtained from the calibration table for blood accordingly.

1. In a 50 ml volumetric flask place
   - 1 ml thoroughly mixed unfiltered urine from an Ostwald-Folin pipette
   - 4 ml 0.4% uranium acetate (13) and shake thoroughly for 15 seconds or until foam-free.
2. Allow to stand for 10 minutes.
3. Dilute to volume with sulfate-free distilled water (1) and filter through a Whatman #40 paper—the filtrate must be crystal clear.
4. In a 15 ml glass-stoppered centrifuge tube with sharply pointed tip place
   - 1 ml filtrate
   - 2 ml 10% trichloracetic acid (2) and mix.
5. Proceed exactly as outlined in the Test Procedure for blood, Steps 12–27 inclusive.
6. Measure the transmittancy of the sample against the reagent blank set at 100% transmission at a wave length of **575 mμ** and multiply the value from the calibration table for blood by 50 to obtain the concentration of inorganic sulfate in mg per 100 ml urine.

Calculation

$$\frac{\text{Volume of 24-hour specimen}}{100} \times \frac{\text{Inorganic sulfate in mg per 100 ml}}{1000} = \text{Gm inorganic sulfate excreted in 24 hours}$$

$$\frac{\text{Gm inorganic sulfate excreted in 24 hours}}{3} = \text{Gm inorganic sulfate-sulfur excreted in 24 hours}$$

PROCEDURE FOR USE WITH LINEAR-SCALE PHOTOELECTRIC COLORIMETERS (SHEARD-SANFORD TYPE)

TEST PROCEDURE

Exactly as outlined; set the reagent blank at 100 and use a green filter.

CALIBRATION PROCEDURE

Exactly as outlined—use a green filter; plot the observed readings on semi-log graph paper—the points fall on a straight line passing through the origin.

PROCEDURE FOR USE WITH LOGARITHMIC-SCALE PHOTOELECTRIC COLORIMETERS (KLETT-SUMMERSON TYPE)

TEST PROCEDURE

Exactly as outlined; set the reagent blank at 0 and use a green filter.

CALIBRATION PROCEDURE

Exactly as outlined—use a green filter; plot the observed readings on linear graph paper—the points fall on a straight line passing through the origin.

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NOTES

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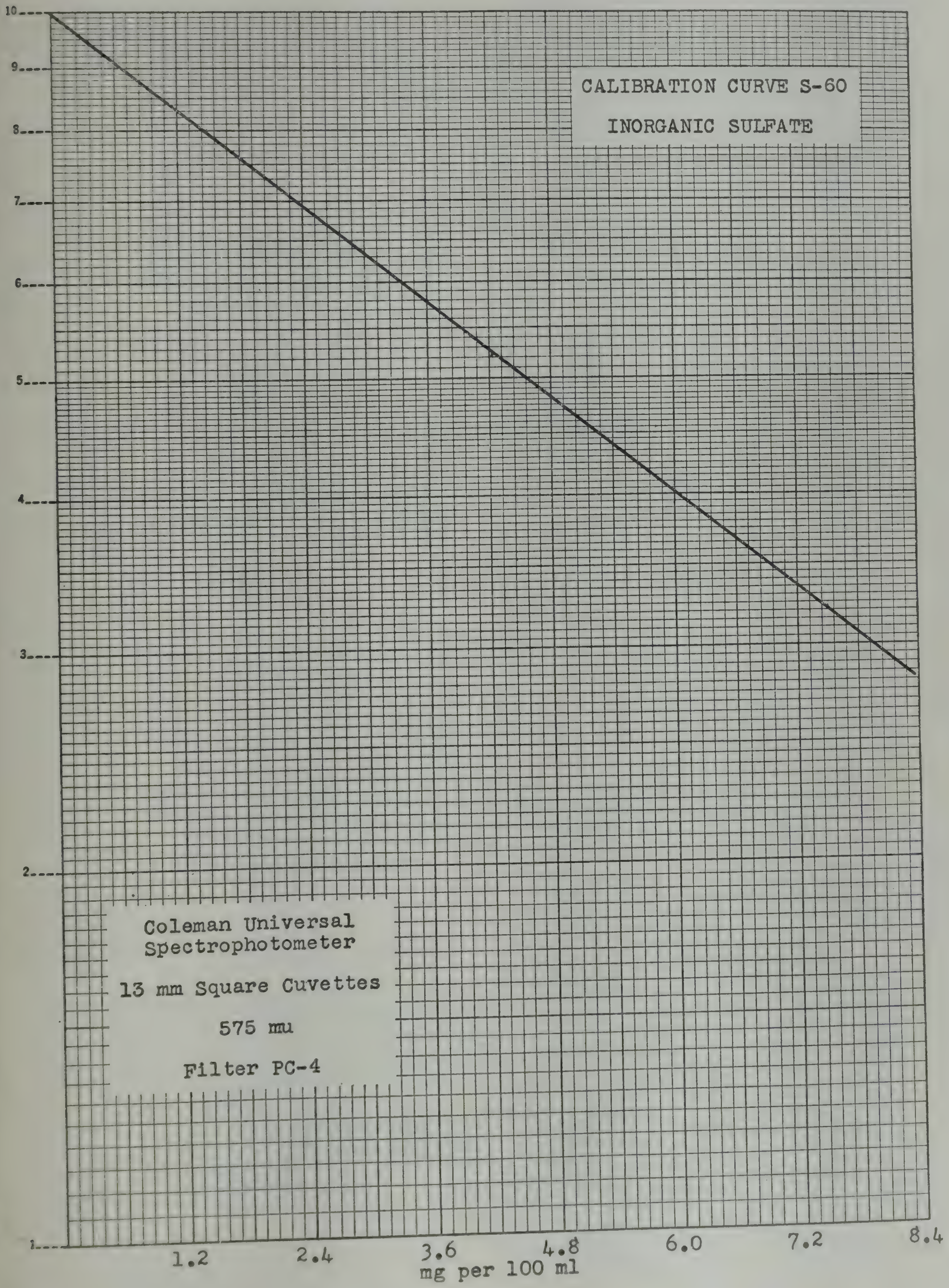

CALIBRATION CURVE S-60

INORGANIC SULFATE

Coleman Universal
Spectrophotometer

13 mm Square Cuvettes

575 mu

Filter PC-4

Meter Reading	0	1	2	3	4	5	6	7	8	9
0										
10										
20										
30										
40										
50										
60										
70										
80										
90										

SULFATE, TOTAL

A spectrophotometric adaptation based on the methods and modifications of Yoshimatsu (1926), Hubbard (1927), Kahn and Leiboff (1928), Wakefield (1929), Letonoff and Reinhold (1936), Marenzi and Banfi (1939) and Klein (1944)

PRINCIPLE

Neutral sulfur and ethereal sulfates are converted to inorganic sulfate by oxidation and hydrolysis, and together with the inorganic sulfate originally present, constitute total sulfate. The sulfate is then determined as outlined in the method for inorganic sulfate (Method S-60), the same analytical principles being involved.

DETERMINATION IN WHOLE BLOOD, PLASMA OR SERUM

SAMPLE

Blood withdrawn during post-absorptive state. The freshly drawn blood must be defibrinated by vigorous stirring with a glass rod; fluorides, oxalates or heparin cannot be used as anticoagulants. Analyses preferably made on serum.

TEST PROCEDURE

Special Apparatus

Ostwald-Folin pipettes accurately calibrated "to deliver" 4 ml.

Centrifuge tubes, 15 ml, Pyrex, glass-stoppered, with sharply pointed tips.

Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.

Note: Glassware must be absolutely free of sulfates. Dichromate-sulfuric acid solution should not be used for cleaning—instead, use a mixture of 300 ml concentrated nitric acid and 1000 ml 5% potassium dichromate; rinse thoroughly with distilled water and dry before use.

Reagents

Note: The distilled water and reagents must be sulfate-free and should show no discernible precipitate with barium chloride.

1. **Water, Distilled, Sulfate-Free.** Redistil distilled water from an all-glass Pyrex distillation apparatus and test for the presence of sulfate with barium chloride. This must be used for the preparation of all reagents and standards and where indicated in the test procedure.
2. **Trichloracetic Acid, 10% Aqueous.** Dilute 10 ml concentrated trichloracetic acid (a) to 100 ml with sulfate-free distilled water (1). Preserve in Pyrex glass-stoppered bottle. Prepare fresh weekly.
   (a) Trichloracetic acid, concentrated; dissolve 100 gm crystalline trichloracetic acid (CCl_3-$COOH$—Baker & Adamson, Reagent) in sulfate-free distilled water (1) and dilute to 100 ml. Preserve in Pyrex glass-stoppered bottle. Stability indefinite. Note: Trichloracetic acid, even of the grade specified, always contains traces of sulfate. It must be purified by adding 5 ml 0.5% benzidine to the molten acid and re-distilling in vacuo at a pressure of 15–20 mm.
3. **Hydrochloric Acid, Volumetric 1 N.** Slowly add 100 ml concentrated hydrochloric acid (HCl—Baker & Adamson, A.C.S.) to 1000 ml sulfate-free distilled water (1) and mix; standardize against 1 N alkali using phenolphthalein as indicator.
4. **Aluminum Chloride, 1.38% Aqueous.** Dissolve 1.38 gm anhydrous aluminum chloride ($AlCl_3$—Mallinckrodt, AR) in sulfate-free distilled water (1) and dilute to 100 ml. Note: In place of anhydrous aluminum chloride, 2.5 gm of the crystalline form may be dissolved in 100 ml water—the anhydrous form is preferred because of its much lower sulfate content.
5. **Ammonia Solution.** Dilute 20 ml ammonium hydroxide (NH_4OH—Baker & Adamson, A.C.S.) to 100 ml with sulfate-free distilled water (1) and add 5 ml 0.02% methyl red indicator solution (a).
   (a) Methyl red indicator solution, 0.02% aqueous; dissolve 10.8 mg of the sodium salt of methyl red ($C_{15}H_{14}O_2N_3Na$—Coleman & Bell, Water soluble) in sulfate-free distilled water (1) and dilute to 50 ml.
6. **Trichloracetic Acid, 5% in Acetone (Marenzi-Banfi).** Add 5 gm crystalline re-distilled trichloracetic acid (see note under Reagent #2a) to 100 ml re-distilled anhydrous acetone ($(CH_3)_2CO$—Baker & Adamson, A.C.S.) and shake until dissolved. Preserve in Pyrex glass-stoppered bottle. Prepare fresh daily. Solutions showing a yellow tinge should be discarded.
7. **Benzidine, 1.5% in Acetone (Marenzi-Banfi).** Add 1.5 gm benzidine ($(C_6H_4)_2(NH_2)_2$—Matheson) to 100 ml re-distilled anhydrous acetone ($(CH_3)_2CO$—Baker & Adamson, A.C.S.) and shake until dissolved; filter, if necessary, through a Whatman #40 paper. Prepare fresh daily.
8. **Hydrochloric Acid, Volumetric 0.2 N.** Dilute 20 ml 1 N hydrochloric acid (3) to 100 ml with sulfate-free distilled water (1) and mix.
9. **Sodium Nitrite, 0.1% Aqueous.** Dilute 1 ml 10% sodium nitrite (a) to 100 ml with sulfate-free distilled water (1). Prepare fresh daily and preserve in a brown glass bottle.
   (a) Sodium nitrite, 10% aqueous; dissolve 10

gm granular sodium nitrite ($NaNO_2$—Mallinckrodt, A.C.S.) in sulfate-free distilled water (1) and dilute to 100 ml. Preserve in refrigerator.

10. **Ammonium Sulfamate, 0.5% Aqueous.** Dissolve 0.5 gm crystalline ammonium sulfamate ($NH_2SO_3NH_4$—Reagent Chemicals, Special) in sulfate-free distilled water (1) and dilute to 100 ml. Stable indefinitely; discard if mold-growth occurs.
11. **N-(1-Naphthyl)-Ethylenediamine Dihydrochloride, 0.1% Aqueous.** Dissolve 100 mg N-(1-naphthyl)-ethylenediamine dihydrochloride ($C_{10}H_7HNCH_2CH_2NH_2 \cdot 2HCl$—Eastman #4835) in 100 ml sulfate-free distilled water (1). Preserve in a brown glass bottle in refrigerator. Stability about 1 month. Discard when a brownish color appears.

Technic

Note: For concentrations of total sulfate in excess of 15 mg per 100 ml, add a larger volume of 0.2 N hydrochloric acid (8) in Step 20, use an aliquot and correct the values obtained from the calibration table for inorganic sulfate accordingly.

1. In a 15 ml centrifuge tube place
   8 ml 10% trichloracetic acid (2)
   4 ml sample from an Ostwald-Folin pipette, added drop by drop, while constantly agitating the tube.
2. Shake vigorously for 1 minute and allow to stand for 20 minutes.
3. Centrifuge at 1500 rpm for 5 minutes.
4. In a 30 ml beaker place
   6 ml clear centrifugate
   0.5 ml 1 N hydrochloric acid (3).
5. Evaporate to dryness on a water bath.
6. Allow to cool to room temperature and dissolve the residue in 6 ml sulfate-free distilled water (1).
7. Add 0.5 ml 1.38% aluminum chloride (4) and mix thoroughly by stirring with a glass rod.
8. Add 0.5 ml ammonia solution (5) and mix—distinct alkalinity is evidenced by a yellow color but an excess of ammonia must be avoided.
9. Allow to stand for 10 minutes until the aluminum hydroxide flocculates.
10. Transfer to a 15 ml centrifuge tube and centrifuge at 2000 rpm for 5 minutes.
11. Transfer 3.5 ml clear centrifugate to a 15 ml glass-stoppered centrifuge tube with sharply pointed tip.
12. Neutralize by the addition of freshly prepared 5% trichloracetic acid in acetone (6)—about 1 ml is required.
13. Add an additional 2 ml 5% trichloracetic acid in acetone (6)—the solution must now be distinctly acid as evidenced by a red color.
14. Add 6 ml freshly prepared 1.5% benzidine in acetone (7) and mix.
15. Stopper the tube and place in the refrigerator at 0° C. for 2 hours—longer does no harm.
16. Centrifuge the stoppered tube at 3000 rpm for 15 minutes.
17. Decant the supernatant fluid and discard; drain the tube for 3 minutes by inverting on a filter paper moistened with acetone.
18. Break up the precipitate with a thin glass rod and add 10 ml 5% trichloracetic acid in acetone (6) washing down the walls of the tube and rinsing off the rod at the same time.
19. Centrifuge and drain for 5 minutes as in Steps 16 and 17; wipe the lip of the tube dry with a tissue.
20. Add exactly 4 ml 0.2 N hydrochloric acid (8) and place the tube in a water bath at 60° C. for 5 minutes, shaking frequently, until the precipitate is dissolved—the contents must not be allowed to boil.
21. Cool to room temperature and transfer 2 ml to an 18 × 150 mm test tube (sample).
22. Add 6 ml sulfate-free distilled water (1) and mix.
23. In another 18 × 150 mm test tube (reagent blank) place 8 ml sulfate-free distilled water (1).
24. Place both tubes in a beaker half-filled with ice cold water.
25. To each tube add 1 ml 0.1% sodium nitrite (9), mix and allow to remain in the cold water for 3 minutes.
26. Add 1 ml 0.5% ammonium sulfamate (10), mix and allow to remain in the cold water for 2 minutes.
27. Remove the tubes from the bath and add 1 ml 0.1% N-(1-naphthyl)-ethylenediamine dihydrochloride (11) and mix thoroughly.
28. Allow to stand at room temperature for 20 minutes.
29. Transfer the contents of the tubes to correspondingly marked 100 ml volumetric flasks, rinsing out the tubes with several small portions sulfate-free distilled water (1) and adding the washings to the flasks.
30. Dilute to volume with sulfate-free distilled water (1) and mix thoroughly by inversion.
31. Measure the transmittancy of the sample against the reagent blank set at 100% transmission at a wave length of **575 mμ** and multiply the value from the calibration table for inorganic sulfate (Method S-60) by 2 to obtain the concentration of total sulfate in mg per 100 ml blood.

CALIBRATION PROCEDURE

This calibration procedure need not be carried out—the values in the calibration table for inorganic sulfate (Method S-60), when multiplied by 2, are equivalent to the concentration of total sulfate in mg per 100 ml sample.

CONVERSION FACTOR

$$\frac{\text{Total sulfate in mg per 100 ml}}{3} = \text{Total sulfate-sulfur in mg per 100 ml}$$

Method S-61b

DETERMINATION IN URINE

PRINCIPLE

Phosphates are removed by treatment with uranium acetate; neutral sulfur and ethereal sulfates are converted to inorganic sulfate which is then determined as outlined for blood, the same analytical principles being involved.

SAMPLE

Aliquot of a 24-hour specimen. The greater the 24-hour volume, the larger the aliquot to be taken for analysis. For normal urines a 1:100 dilution is usually sufficient.

TEST PROCEDURE

Special Apparatus

Ostwald-Folin pipettes accurately calibrated "to deliver" 1 ml.
Centrifuge tubes, 15 ml, Pyrex, glass-stoppered, with sharply pointed tips.
Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.

Reagents

1. **Water, Distilled, Sulfate-Free.** Same as for blood.
3. **Hydrochloric Acid, Volumetric 1 N.** Same as for blood.
6. **Trichloracetic Acid, 5% in Acetone (Marenzi-Banfi).** Same as for blood.
7. **Benzidine, 1.5% in Acetone (Marenzi-Banfi).** Same as for blood.
8. **Hydrochloric Acid, Volumetric 0.2 N.** Same as for blood.
9. **Sodium Nitrite, 0.1% Aqueous.** Same as for blood.
10. **Ammonium Sulfamate, 0.5% Aqueous.** Same as for blood.
11. **N-(1-Naphthyl)-Ethylenediamine Dihydrochloride, 0.1% Aqueous.** Same as for blood.
12. **Uranium Acetate, 0.4% Aqueous.** Dissolve 0.4 gm crystalline uranium acetate ($(CH_3COO)_2$-$UO_2 \cdot 2H_2O$—J. T. Baker, CP Analyzed) in sulfate-free distilled water (1) and dilute to 100 ml.

Technic

Note: For concentrations of total sulfate in excess of 750 mg per 100 ml, dilute 1 ml urine to 200 ml or even more with sulfate-free distilled water (1) in Step 1 and correct the values from the calibration table for inorganic sulfate (Method S-60) accordingly.

1. In a 100 ml volumetric flask place
   1 ml thoroughly mixed unfiltered urine from an Ostwald-Folin pipette
   4 ml 0.4% uranium acetate (12) and shake thoroughly for 15 seconds or until foam-free.
2. Allow to stand for 10 minutes.
3. Dilute to volume with sulfate-free distilled water (1) and filter through a Whatman #40 paper—the filtrate must be crystal clear.
4. In a 30 ml beaker place
   2 ml filtrate
   2 ml sulfate-free distilled water (1)
   0.5 ml 1 N hydrochloric acid (3).
5. Evaporate to dryness on a water bath.
6. Allow to cool to room temperature and dissolve the residue in 2 ml sulfate-free distilled water (1).
7. Transfer the contents of the beaker to a 15 ml glass-stoppered centrifuge tube with sharply pointed tip, rinsing out the beaker with two 1 ml portions sulfate-free distilled water (1), adding the washings to the centrifuge tube.
8. Add 2 ml 5% trichloracetic acid in acetone (6) and mix.
9. Proceed exactly as outlined in the Test Procedure for blood, Steps 14–30 inclusive.
10. Measure the transmittancy of the sample against the reagent blank set at 100% transmission at a wave length of 575 mμ and multiply the value from the calibration table for inorganic sulfate (Method S-60) by 100 to obtain the concentration of total sulfate in mg per 100 ml urine.

Calculation

$$\frac{\text{Volume of 24-hour specimen}}{100} \times \frac{\text{Total sulfate in mg per 100 ml}}{1000} = \text{Gm total sulfate excreted in 24 hours}$$

$$\frac{\text{Gm total sulfate excreted in 24 hours}}{3} = \text{Gm total sulfate-sulfur excreted in 24 hours}$$

PROCEDURE FOR USE WITH LINEAR-SCALE PHOTOELECTRIC COLORIMETERS (SHEARD-SANFORD TYPE)

TEST PROCEDURE

Exactly as outlined; set the reagent blank at 100 and use a green filter.

CALIBRATION PROCEDURE

Need not be performed; values obtained from calibration table for inorganic sulfate.

PROCEDURE FOR USE WITH LOGARITHMIC-SCALE PHOTOELECTRIC COLORIMETERS (KLETT-SUMMERSON TYPE)

TEST PROCEDURE

Exactly as outlined; set the reagent blank at 0 and use a green filter.

CALIBRATION PROCEDURE

Need not be performed; values obtained from calibration table for inorganic sulfate.

BIBLIOGRAPHY

Same as listed under Inorganic Sulfate (Method S-60).

NOTES

SULFATE, ETHEREAL

Ethereal sulfate may be determined in whole blood plasma, serum or urine as follows:

1. Determine total sulfate as outlined in Method S-61.
2. Determine inorganic sulfate as outlined in Method S-60.
3. Calculation—
   Total sulfate in mg per 100 ml − Inorganic sulfate in mg per 100 ml = Ethereal sulfate in mg per 100 ml

   $$\frac{\text{Ethereal sulfate in mg per 100 ml}}{3} = \text{Ethereal sulfate-sulfur in mg per 100 ml}$$

NOTES

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SULFUR, TOTAL

A spectrophotometric adaptation based on the methods and modifications of Yoshimatsu (1926), Hubbard (1927), Kahn and Lieboff (1928), Wakefield (1929), Letonoff and Reinhold (1936), Marenzi and Banfi (1939) and Klein (1944)

PRINCIPLE

Organic matter in the sample is destroyed by digestion with nitric acid and hydrogen peroxide and the total sulfur determined as outlined in the method for inorganic sulfate (Method S-60), the same analytical principles being involved.

DETERMINATION IN WHOLE BLOOD, PLASMA OR SERUM

SAMPLE

Blood withdrawn during post-absorptive state. The freshly drawn blood must be defibrinated by vigorous stirring with a glass rod; fluorides, oxalates or heparin cannot be used as anticoagulants. Analyses preferably made on serum.

TEST PROCEDURE

Special Apparatus

Ostwald-Folin pipettes accurately calibrated "to deliver" 4 ml.
Kjeldahl flasks, 100 ml, Pyrex.
Centrifuge tubes, 15 ml, Pyrex, glass-stoppered, with sharply pointed tips.
Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.

Note: Glassware must be absolutely free of sulfates. Dichromate-sulfuric acid solution should not be used for cleaning—instead, use a mixture of 300 ml concentrated nitric acid and 1000 ml 5% potassium dichromate; rinse thoroughly with distilled water and dry before use.

Reagents

Note: The distilled water and reagents must be sulfate-free and should show no discernible precipitate with barium chloride.

1. **Water, Distilled, Sulfate-Free.** Redistil distilled water from an all-glass Pyrex distillation apparatus and test for the presence of sulfate with barium chloride. This must be used for the preparation of all reagents and standards and where indicated in the test procedure.
2. **Trichloracetic Acid, 10% Aqueous.** Dilute 10 ml concentrated trichloracetic acid (a) to 100 ml with sulfate-free distilled water (1). Preserve in Pyrex glass-stoppered bottle. Prepare fresh weekly.
   (a) Trichloracetic acid, concentrated; dissolve 100 gm crystalline trichloracetic acid (CCl_3-COOH—Baker & Adamson, Reagent) in sulfate-free distilled water (1) and dilute to 100 ml. Preserve in Pyrex glass-stoppered bottle. Stability indefinite. Note: Trichloracetic acid, even of the grade specified, always contains traces of sulfate. It must be purified by adding 5 ml 0.5% benzidine to the molten acid and re-distilling in vacuo at a pressure of 15–20 mm.
3. **Nitric Acid, Concentrated** (HNO_3—Grasselli, A.C.S.).
4. **Hydrogen Peroxide, 30%** (H_2O_2—Merck, Reagent, Superoxol).
5. **Aluminum Chloride, 1.38% Aqueous.** Dissolve 1.38 gm anhydrous aluminum chloride ($AlCl_3$—Mallinckrodt, AR) in sulfate-free distilled water (1) and dilute to 100 ml. Note: In place of anhydrous aluminum chloride, 2.5 gm of the crystalline form may be dissolved in 100 ml water—the anhydrous form is preferred because of its much lower sulfate content.
6. **Ammonia Solution.** Dilute 20 ml ammonium hydroxide (NH_4OH—Baker & Adamson, A.C.S.) to 100 ml with sulfate-free distilled water (1) and add 5 ml 0.02% methyl red indicator solution (a).
   (a) Methyl red indicator solution, 0.02% aqueous; dissolve 10.8 mg of the sodium salt of methyl red ($C_{15}H_{14}O_2N_3Na$—Coleman & Bell, Water soluble) in sulfate-free distilled water (1) and dilute to 50 ml.
7. **Trichloracetic Acid, 5% in Acetone (Marenzi-Banfi).** Add 5 gm crystalline re-distilled trichloracetic acid (see note under Reagent #2a) to 100 ml re-distilled anhydrous acetone ($(CH_3)_2CO$—Baker & Adamson, A.C.S.) and shake until dissolved. Preserve in Pyrex glass-stoppered bottle. Prepare fresh daily. Solutions showing a yellow tinge should be discarded.
8. **Benzidine, 1.5% in Acetone (Marenzi-Banfi).** Add 1.5 gm benzidine ($(C_6H_4)_2(NH_2)_2$—Matheson) to 100 ml re-distilled anhydrous acetone ($(CH_3)_2CO$—Baker & Adamson, A.C.S.) and shake until dissolved; filter, if necessary, through a Whatman #40 paper. Prepare fresh daily.
9. **Hydrochloric Acid, Volumetric 0.2 N.** Slowly add 17 ml concentrated hydrochloric acid (HCl—Baker & Adamson, A.C.S.) to 1000 ml sulfate-free distilled water (1) and mix well; standardize against 0.2 N alkali using phenolphthalein as indicator.
10. **Sodium Nitrite, 0.1% Aqueous.** Dilute 1 ml 10% sodium nitrite (a) to 100 ml with sulfate-

free distilled water (1). Prepare fresh daily and preserve in a brown glass bottle.

   (a) Sodium nitrite, 10% aqueous; dissolve 10 gm granular sodium nitrite ($NaNO_2$—Mallinckrodt, A.C.S.) in sulfate-free distilled water (1) and dilute to 100 ml. Preserve in refrigerator.

11. **Ammonium Sulfamate, 0.5% Aqueous.** Dissolve 0.5 gm crystalline ammonium sulfamate ($NH_2SO_3NH_4$—Reagent Chemicals, Special) in sulfate-free distilled water (1) and dilute to 100 ml. Stable indefinitely; discard if mold-growth occurs.
12. **N-(1-Naphthyl)-Ethylenediamine Dihydrochloride, 0.1% Aqueous.** Dissolve 100 mg N-(1-naphthyl)-ethylenediamine dihydrochloride ($C_{10}H_7HNCH_2CH_2NH_2 \cdot 2HCl$—Eastman #4835) in 100 ml sulfate-free distilled water (1). Preserve in a brown glass bottle in refrigerator. Stability about 1 month. Discard when a brownish color appears.

Technic

Note: For concentrations of total sulfur in excess of 10 mg per 100 ml, add a larger volume of 0.2 N hydrochloric acid (9) in Step 23, use an aliquot and correct the values obtained from the calibration tables for inorganic sulfate (Method S-60) or total sulfur accordingly.

1. In a 15 ml centrifuge tube place
   8 ml 10% trichloracetic acid (2)
   4 ml sample from an Ostwald-Folin pipette, added drop by drop, while constantly agitating the tube.
2. Shake vigorously for 1 minute and allow to stand for 20 minutes.
3. Centrifuge at 1500 rpm for 5 minutes.
4. In a 100 ml Pyrex Kjeldahl flask place
   1.5 ml clear centrifugate
   1.5 ml sulfate-free distilled water (1)
   3 ml concentrated nitric acid (3).
5. Heat over a free flame until the volume of the digest is less than 1 ml and has a brownish color.
6. Cool for 30 seconds and allow 2 drops 30% hydrogen peroxide (4) to fall directly into the digest.
7. Heat for 1 minute—if the digest is not clear, add more peroxide, reheat, and continue this process until the digest clears.
8. Heat with a full flame to a white ash.
9. Allow to cool to room temperature and dissolve the residue in 6 ml sulfate-free distilled water (1).
10. Add 0.5 ml 1.38% aluminum chloride (5) and mix.
11. Add 0.5 ml ammonia solution (6) and mix—distinct alkalinity is evidenced by a yellow color but an excess of ammonia must be avoided.
12. Allow to stand for 10 minutes until the aluminum hydroxide flocculates.
13. Transfer the contents of the flask to a 15 ml centrifuge tube and centrifuge at 2000 rpm for 5 minutes.
14. Transfer 3.5 ml clear centrifugate to a 15 ml glass-stoppered centrifuge tube with sharply pointed tip.
15. Neutralize by the addition of freshly prepared 5% trichloracetic acid in acetone (7)—about 1 ml is required.
16. Add an additional 2 ml 5% trichloracetic acid in acetone (7)—the solution must now be distinctly acid as evidenced by a red color.
17. Add 6 ml freshly prepared 1.5% benzidine in acetone (8) and mix.
18. Stopper the tube and place in the refrigerator at 0° C. for 2 hours—longer does no harm.
19. Centrifuge the stoppered tube at 3000 rpm for 15 minutes.
20. Decant the supernatant fluid and discard; drain the tube for 3 minutes by inverting on a filter paper moistened with acetone.
21. Break up the precipitate with a thin glass rod and add 10 ml 5% trichloracetic acid in acetone (7), washing down the walls of the tube and rinsing off the rod at the same time.
22. Centrifuge and drain for 5 minutes as in Steps 19 and 20; wipe the lip of the tube dry with a tissue.
23. Add 2 ml 0.2 N hydrochloric acid (9) and place the tube in a water bath at 60° C. for 5 minutes shaking frequently, until the precipitate is dissolved—the contents must not be allowed to boil.
24. Cool to room temperature and transfer the contents of the tube to an 18 × 150 mm test tube (sample), rinsing the centrifuge tube with three 1–2 ml portions sulfate-free distilled water (1), adding the washings to the test tube, bringing the volume to 6–8 ml.
25. In another 18 × 150 mm test tube (reagent blank) place 6–8 ml sulfate-free distilled water (1).
26. Place both tubes in a beaker half-filled with ice cold water.
27. To each tube add 1 ml 0.1% sodium nitrite (10), mix and allow to remain in the cold water for 3 minutes.
28. Add 1 ml 0.5% ammonium sulfamate (11), mix and allow to remain in the cold water for 2 minutes.
29. Remove the tubes from the bath and add 1 ml 0.1% N-(1-naphthyl)-ethylenediamine dihydrochloride (12) and mix thoroughly.
30. Allow to stand at room temperature for 20 minutes.
31. Transfer the contents of the tubes to correspondingly marked 100 ml volumetric flasks, rinsing out the tubes with several small portions sulfate-free distilled water (1) and adding the washings to the flasks.
32. Dilute to volume with sulfate-free distilled water (1) and mix thoroughly by inversion.
33. Measure the transmittancy of the sample against the reagent blank set at 100% transmission at a wave length of 575 mμ and divide the value from the calibration table for inorganic sulfate (Method S-60) by 0.75 to obtain the concentration of total sulfur in mg per 100 ml sample. If a calibration

table has been prepared for total sulfur, the concentration is obtained directly.

CALIBRATION PROCEDURE

This calibration procedure need not be carried out. A calibration curve (Calibration Curve S-63) may be constructed by substituting the values 2, 4, 6, 8 and 10 mg sulfur per 100 ml for the transmittancy readings recorded for the 1.5, 3.0, 4.5, 6.0 and 7.5 mg sulfate per 100 ml concentrations in the calibration curve for inorganic sulfate (Method S-60).

Method S-63b

DETERMINATION IN URINE

PRINCIPLE

Phosphates are removed by treatment with uranium acetate, the organic matter is destroyed and total sulfur is determined as outlined for blood, the same analytical principles being involved.

SAMPLE

Aliquot of a 24-hour specimen. The greater the 24-hour volume, the larger the aliquot to be taken for analysis. For normal urines a 1:100 dilution is usually sufficient.

TEST PROCEDURE

Special Apparatus

Ostwald-Folin pipettes accurately calibrated "to deliver" 1 ml.
Kjeldahl flasks, 100 ml, Pyrex.
Centrifuge tubes, 15 ml, Pyrex, glass-stoppered, with sharply pointed tips.
Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.

Reagents

1. **Water, Distilled, Sulfate-Free.** Same as for blood.
2. **Trichloracetic Acid, 10% Aqueous.** Same as for blood.
3. **Nitric Acid, Concentrated.** Same as for blood.
4. **Hydrogen Peroxide, 30%.** Same as for blood.
7. **Trichloracetic Acid, 5% in Acetone (Marenzi-Banfi).** Same as for blood.
8. **Benzidine, 1.5% in Acetone (Marenzi-Banfi).** Same as for blood.
9. **Hydrochloric Acid, Volumetric 0.2 N.** Same as for blood.
10. **Sodium Nitrite, 0.1% Aqueous.** Same as for blood.
11. **Ammonium Sulfamate, 0.5% Aqueous.** Same as for blood.
12. **N-(1-Naphthyl)-Ethylenediamine Dihydrochloride, 0.1% Aqueous.** Same as for blood.
13. **Uranium Acetate, 0.4% Aqueous.** Dissolve 0.4 gm crystalline uranium acetate ($(CH_3COO)_2UO_2 \cdot 2H_2O$—J. T. Baker, CP Analyzed) in sulfate-free distilled water (1) and dilute to 100 ml.

Technic

Note: For concentrations of total sulfur in excess of 500 mg per 100 ml, dilute 1 ml urine to 200 ml or even more with sulfate-free distilled water (1) in Step 1 and correct the values obtained from the calibration tables for inorganic sulfate in blood (Method S-60) or total sulfur in blood accordingly.

1. In a 100 ml volumetric flask place
   1 ml thoroughly mixed unfiltered urine from an Ostwald-Folin pipette
   4 ml 0.4% uranium acetate (13) and shake thoroughly for 15 seconds or until foam-free.
2. Allow to stand for 10 minutes.
3. Dilute to volume with sulfate-free distilled water (1) and filter through a Whatman #40 paper—the filtrate must be crystal clear.
4. In a 100 ml Pyrex Kjeldahl flask place
   1 ml filtrate
   3 ml concentrated nitric acid (3).
5. Heat over a free flame until the volume of the digest is less than 1 ml and has a brownish color.
6. Cool for 30 seconds and allow 2 drops 30% hydrogen peroxide (4) to fall directly into the digest.
7. Heat for 1 minute—if the digest is not clear, add more peroxide, reheat, and continue this process until the digest clears.
8. Heat with a full flame to a white ash.
9. Allow to cool to room temperature and dissolve the residue in 6 ml 10% trichloracetic acid (2).
10. Transfer the contents of the flask to a 15 ml centrifuge tube and centrifuge at 2000 rpm for 5 minutes.
11. Transfer 3 ml clear centrifugate to a 15 ml glass-stoppered centrifuge tube with sharply pointed tip.
12. Add 2 ml 5% trichloracetic acid in acetone (7) and mix.
13. Proceed exactly as outlined in the Test Procedure for blood, Steps 17–32 inclusive.
14. Measure the transmittancy of the sample against the reagent blank set at 100% transmission at a wave length of 575 mμ and divide the value from the calibration table for inorganic sulfate in blood (Method S-60) by 0.015 to obtain the concentration of total sulfur in mg per 100 ml urine. If a calibration table has been prepared for total sulfur in blood, multiply the value by 50 to obtain the concentration of total sulfur in mg per 100 ml urine.

Calculation

$$\frac{\text{Volume of 24-hour specimen}}{100} \times \frac{\text{Total sulfur in mg per 100 ml}}{1000} = \text{Gm total sulfur excreted in 24 hours}$$

PROCEDURE FOR USE WITH LINEAR-SCALE PHOTOELECTRIC COLORIMETERS (SHEARD-SANFORD TYPE)

TEST PROCEDURE

Exactly as outlined; set the reagent blank at 100 and use a green filter.

CALIBRATION PROCEDURE

Need not be performed. The values may be obtained from the calibration table for inorganic sulfate.

PROCEDURE FOR USE WITH LOGARITHMIC-SCALE PHOTOELECTRIC COLORIMETERS (KLETT-SUMMERSON TYPE)

TEST PROCEDURE

Exactly as outlined; set the reagent blank at 0 and use a green filter.

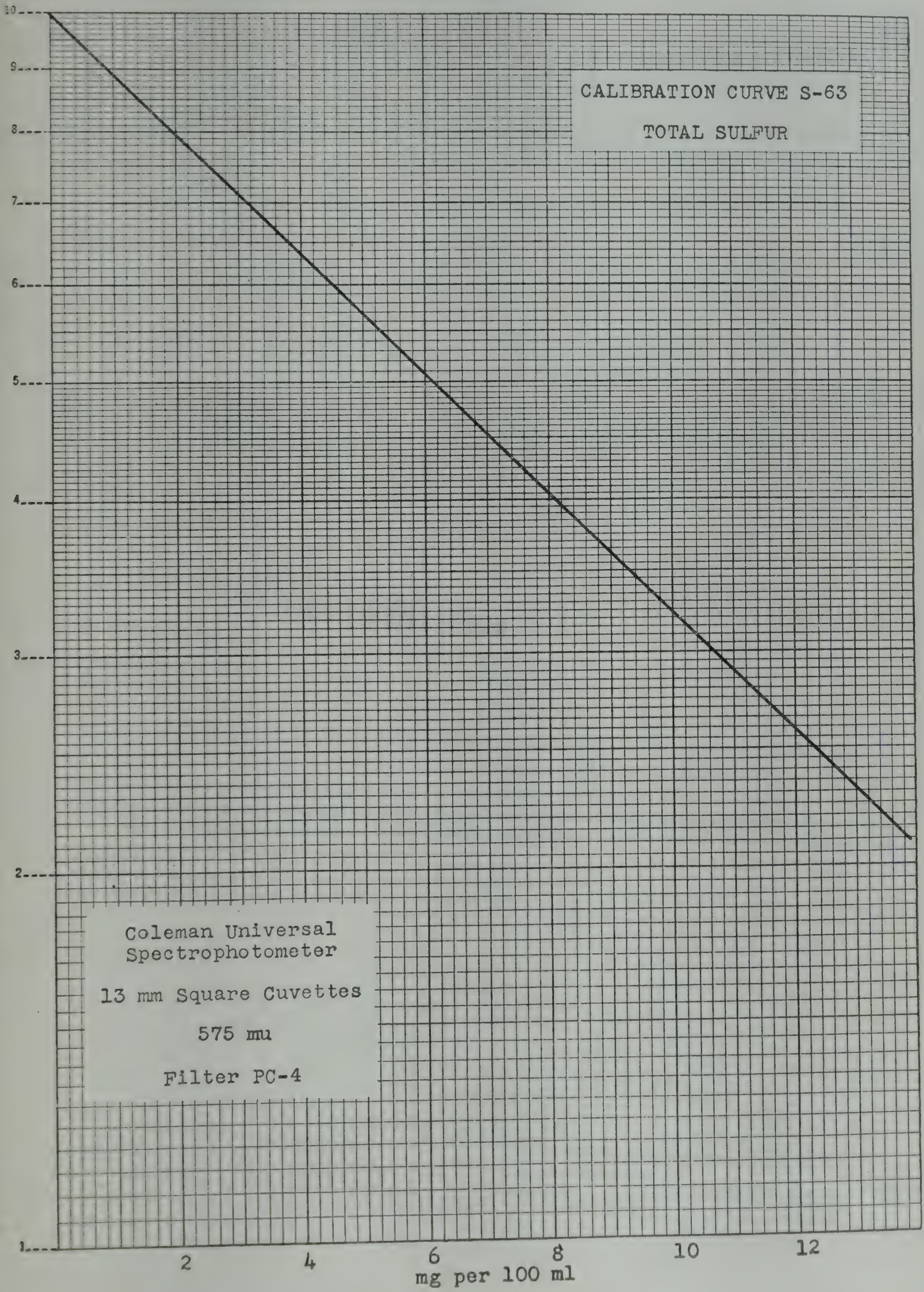
CALIBRATION PROCEDURE

Need not be performed. The values may be obtained from the calibration table for inorganic sulfate.

BIBLIOGRAPHY

Same as listed under Inorganic Sulfate (Method S-60).

NOTES


CALIBRATION CURVE S-63
TOTAL SULFUR
Coleman Universal Spectrophotometer
13 mm Square Cuvettes
575 mu
Filter PC-4
10
9
8
7
6
5
4
3
2
1
2
4
6
8
10
12
mg per 100 ml

Meter Reading	0	1	2	3	4	5	6	7	8	9
0										
10										
20										
30										
40										
50										
60										
70										
80										
90										

SULFUR, NEUTRAL

Neutral sulfur may be determined in whole blood plasma, serum or urine as follows:

1. Determine total sulfur as outlined in Method S-63.
2. Determine total sulfate and convert to terms of sulfur as outlined in Method S-61.
3. Calculation—

Total sulfur in mg per 100 ml − Total sulfate-sulfur in mg per 100 ml = Neutral sulfur in mg per 100 ml

NOTES

NOTES

THIOCYANATE

A spectrophotometric adaptation based on the methods and modifications of Schreiber (1925), Crandall and Anderson (1934), Barker (1936), Lavietes, Bourdillon and Klinghoffer (1936), Bowler (1944), and Powell (1945)

PRINCIPLE

Thiocyanate in a protein-free trichloracetic acid filtrate is treated with ferric nitrate to produce orange-red ferric thiocyanate (Leared reaction).

DETERMINATION IN PLASMA OR SERUM

SAMPLE

Plasma or serum from blood withdrawn during postabsorptive state. Anticoagulant—potassium oxalate; excess oxalate or the use of fluoride or citrate must be avoided. Analyses preferably made on serum. Separate plasma or serum from the red blood cells as soon as possible and store in refrigerator until use. No salicylates or acetylsalicylic acid must be administered to the patient for at least 8 hours prior to the collection of blood.

TEST PROCEDURE

Special Apparatus

Ostwald-Folin pipettes, accurately calibrated "to contain" 1 ml.

Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.

Reagents

1. **Trichloracetic Acid, 20% Aqueous.** Dilute 20 ml concentrated trichloracetic acid (a) to 100 ml with distilled water. Preserve in glass-stoppered bottle. Prepare fresh monthly.
   (a) Trichloracetic acid, concentrated; dissolve 100 gm crystalline trichloracetic acid (CCl_3-$COOH$—J. T. Baker, CP Analyzed) in distilled water and dilute to 100 ml. Preserve in glass-stoppered bottle. Stability indefinite.
2. **Trichloracetic Acid, 10% Aqueous.** Dilute 10 ml concentrated trichloracetic acid (1a) to 100 ml with distilled water. Prepare fresh weekly.
3. **Ferric Nitrate Reagent** (Bowler). Dissolve 80 gm crystalline ferric nitrate ($Fe(NO_3)_3 \cdot 9H_2O$—Baker & Adamson, A.C.S.) in 250 ml 2 N nitric acid (a); transfer to a 500 ml mixing cylinder and dilute to volume with distilled water. Stability indefinite.
   (a) Nitric acid, volumetric 2 N; dilute 130 ml concentrated nitric acid (HNO_3—Grasselli, A.C.S.) to 1000 ml with distilled water; allow to cool; dilute 25 ml of this approximately 2 N solution to 50 ml; titrate against standard 1 N alkali using phenolphthalein as indicator; adjust accordingly and re-titrate—20 ml of a 1:2 dilution of 2 N acid require 20 ml of 1 N alkali. Preserve in glass-stoppered bottle.

Technic

Note: For concentrations of thiocyanate in excess of 100 mg per 100 ml, use 0.5 ml filtrate and 0.5 ml 10% trichloracetic acid (2) in Step 5 and multiply the values obtained from the calibration table by 2.

1. In a 15 ml centrifuge tube place
   4 ml distilled water
   1 ml sample from an Ostwald-Folin pipette thoroughly rinsing out the pipette by drawing in and expelling some of the mixture several times.
2. Add 5 ml 20% trichloracetic acid (1), drop by drop, while constantly agitating the tube.
3. Shake vigorously for 30 seconds and allow to stand for 10 minutes.
4. Centrifuge at 2000 rpm for 10 minutes (or filter through a Schleicher and Schuell #576 paper until crystal clear).
5. In a cuvette (sample) place
   1 ml protein-free centrifugate (or filtrate).
6. In another cuvette (reagent blank) place
   1 ml 10% trichloracetic acid (2).
7. To each cuvette add 4 ml distilled water.
8. Add 5 ml ferric nitrate reagent (3) and mix by inversion.
9. Allow to stand at room temperature for 5 minutes away from direct light.
10. Measure the transmittancy of the sample against the reagent blank set at 100% transmission at a wave length of 465 mμ and obtain the concentration of thiocyanate from the calibration table.

CALIBRATION PROCEDURE

Special Apparatus

Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.

Reagents

2. **Trichloracetic Acid, 10% Aqueous.** Same as for Test Procedure.
3. **Ferric Nitrate Reagent** (Bowler). Same as for Test Procedure.
4. **Silver Nitrate Standard (1 ml equivalent to 1 mg SCN).** Transfer exactly 2.9252 gm finely pulverized crystalline silver nitrate ($AgNO_3$—

Merck, Primary Standard) to a 1000 ml volumetric flask; add 250 ml distilled water and shake until dissolved; add 5 ml concentrated nitric acid (HNO_3—Grasselli, A.C.S.); mix and dilute to volume with distilled water. Preserve in a brown glass bottle.

5. **Trichloracetic Acid, 2% Aqueous.** Dilute 20 ml 10% trichloracetic acid (2) to 100 ml with distilled water. Prepare just before use.
6. **Thiocyanate Standards**
    (a) Thiocyanate stock standard (1 ml contains 1 mg SCN). Dissolve 2 gm crystalline potassium thiocyanate (KSCN—Baker & Adamson, Reagent) in 1000 ml distilled water; titrate against standard silver nitrate (4) using ferric alum as indicator and adjust so that 20 ml potassium thiocyanate are equivalent to 20 ml silver nitrate. Prepare just before use.
    (b) Dilute standard (1 ml contains 0.02 mg SCN). In a 100 ml volumetric flask place 2 ml stock standard (6a) and dilute to volume with 2% trichloracetic acid (5). Prepare just before use.
    (c) Calibration standards. In a series of six cuvettes place respectively 0, 1, 2, 3, 4 and 5 ml of the dilute standard (6b); add 5, 4, 3, 2, 1 and 0 ml 2% trichloracetic acid (5) respectively, bringing the volume in each cuvette to 10 ml. These standards represent concentrations of thiocyanate equivalent to 0, 20, 40, 60, 80 and 100 mg per 100 ml sample. Prepare just before use and proceed immediately as follows.

Technic

1. Treat each calibration standard (6c) exactly as outlined in the Test Procedure, Steps 8–9 inclusive.
2. Set the zero concentration standard at 100% transmission at a wave length of **465** mμ and record the transmittancy of the 20, 40, 60, 80 and 100 mg per cent standards. The transmittancy of the 100 mg standard should be approximately 15%.
3. Plot the observed values on semi-log graph paper—the points fall on a very slight curve which passes through the concentration axis slightly to the right of the origin (Calibration Curve T-10).
4. Prepare a calibration table from the T-C values obtained from the plotted curve.

CONVERSION FACTORS

To convert thiocyanate to potassium thiocyanate multiply by 1.67.

To convert thiocyanate to sodium thiocyanate multiply by 1.39.

◆

PROCEDURE FOR USE WITH LINEAR-SCALE PHOTOELECTRIC COLORIMETERS (SHEARD-SANFORD TYPE)

TEST PROCEDURE

Exactly as outlined; set the reagent blank at 100 and use a blue filter.

CALIBRATION PROCEDURE

Exactly as outlined—use a blue filter; plot the observed readings on semi-log graph paper—the points fall on a straight line passing through the concentration axis slightly to the right of the origin.

◆

PROCEDURE FOR USE WITH LOGARITHMIC-SCALE PHOTOELECTRIC COLORIMETERS (KLETT-SUMMERSON TYPE)

TEST PROCEDURE

Exactly as outlined; set the reagent blank at 0 and use a blue filter.

CALIBRATION PROCEDURE

Exactly as outlined—use a blue filter; plot the observed readings on linear graph paper—the points fall on a straight line passing through the concentration axis slightly to the right of the origin.

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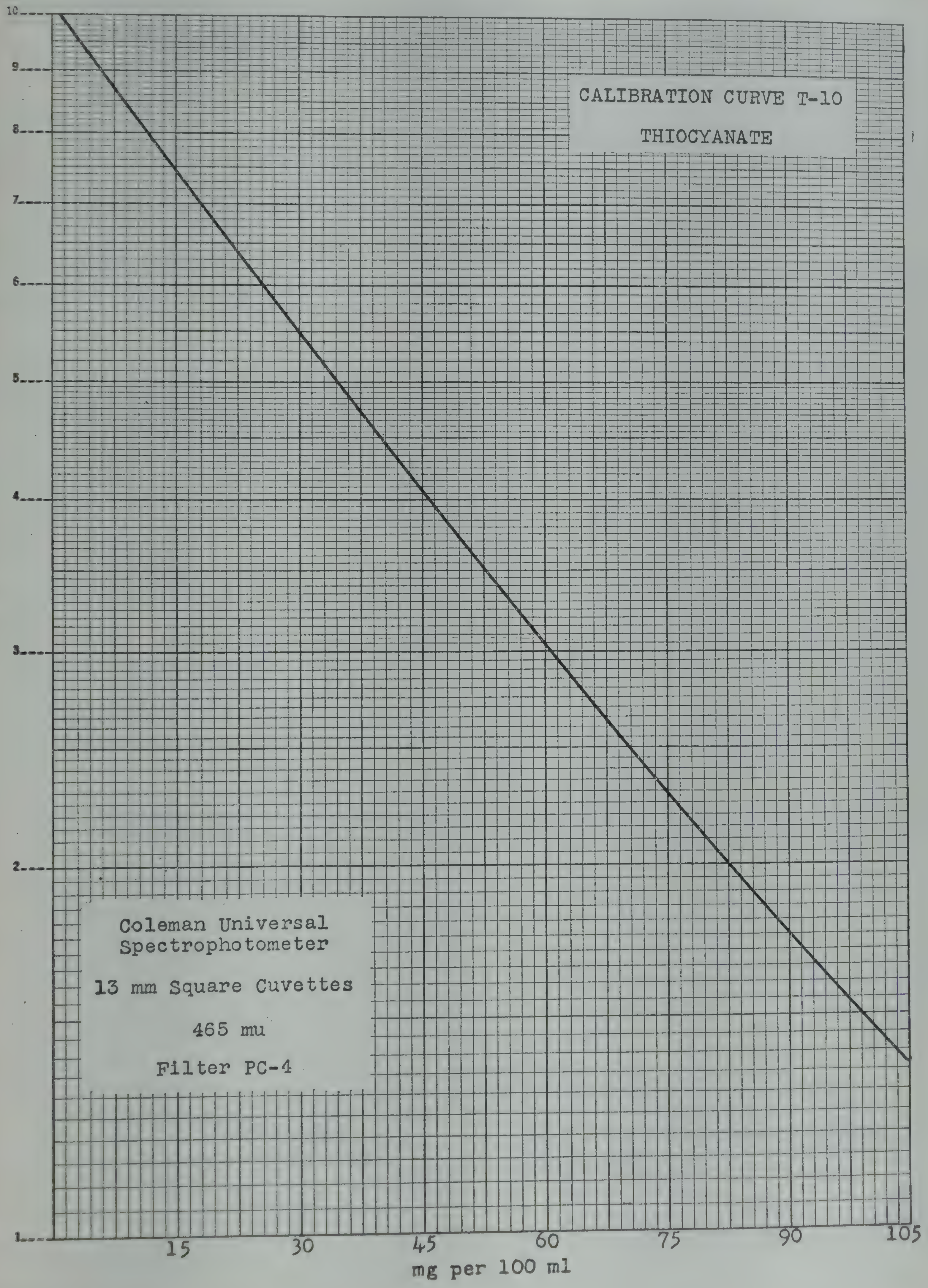
CALIBRATION CURVE T-10
THIOCYANATE
Coleman Universal
Spectrophotometer
13 mm Square Cuvettes
465 mu
Filter PC-4
10
9
8
7
6
5
4
3
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1
15
30
45
60
75
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mg per 100 ml

Meter Reading	0	1	2	3	4	5	6	7	8	9
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THIOURACIL

A spectrophotometric adaptation based on the methods and modifications of Grote (1931), Williams, Jandorf and Kay (1944) and Christensen (1945)

PRINCIPLE

Thiouracil, in a protein-free filtrate, is treated with alkaline nitroferricyanide in the presence of hydroxylamine, at a pH carefully controlled by means of a barbital buffer, to produce an emerald green color (Grote reaction).

DETERMINATION IN WHOLE BLOOD, PLASMA OR SERUM

SAMPLE

Blood withdrawn during post-absorptive state. Anticoagulant for whole blood or plasma—potassium oxalate. Since recovery from whole blood and plasma is approximately only 75%, analyses are preferably made on serum. Samples may be kept in the refrigerator for several days without appreciable change in the thiouracil content.

TEST PROCEDURE

Special Apparatus

Ostwald-Folin pipettes accurately calibrated "to contain" 2 ml.

Graduated test tubes—10 ml in 0.1 ml; the 7.5 ml graduation must be accurately calibrated.

Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.

Reagents

1. **p-Toluenesulfonic Acid, 1 M.** In a 100 ml mixing cylinder place 19 gm crystalline p-toluenesulfonic acid ($CH_3C_6H_4SO_3H \cdot H_2O$—Eastman #984); add 75 ml distilled water and shake until dissolved; dilute to volume; add 1 gm Norit A (Pfanstiehl); shake thoroughly and allow to stand for 1 hour; filter through a Schleicher and Schuell #576 paper until a crystal clear filtrate, free of carbon particles, is obtained. Preserve in a glass-stoppered bottle.
2. **p-Toluenesulfonic Acid, 0.2 M.** Dilute 20 ml 1 M p-toluenesulfonic acid (1) to 100 ml with distilled water.
3. **Thiouracil Standard (2 ml contain 0.2 mg).** This is the same as the dilute thiouracil standard (Reagent #7b). Prepare fresh daily.
4. **Sodium Hydroxide, Volumetric 2.5 N.** Prepare by diluting slightly more than the required amount of concentrated carbonate-free sodium hydroxide solution (see Appendix); dilute 20 ml of this approximately 2.5 N solution to 100 ml; titrate against standard 0.5 N acid using phenolphthalein as indicator; adjust accordingly and re-titrate—20 ml of 0.5 N acid require 20 ml of a 1:5 dilution of 2.5 N alkali. Preserve in ceresin-lined bottle.
5. **Wide Range Indicator Paper** (Hydrion).
6. **Grote Reagent, Buffered (Christensen).** Mix 1 ml Grote reagent (a) with 9 ml barbital buffer solution (b); allow to stand for 1 hour (but no longer) at room temperature (or 25 minutes at 37° C.) before use—during this aging period the color changes from a clear mahogany brown to a light yellow.
   - (a) Grote reagent; in a 50 ml Erlenmeyer flask place 10 ml distilled water; add 0.5 gm sodium nitroprusside ($Na_2Fe(CN)_5(NO) \cdot 2H_2O$—Baker & Adamson, A.C.S.) and shake until dissolved; add 0.5 gm hydroxylamine hydrochloride ($NH_2OH \cdot HCl$—J. T. Baker, CP Analyzed) and shake until dissolved; add 1 gm finely powdered sodium bicarbonate ($NaHCO_3$—Baker & Adamson, A.C.S.); mix by shaking, cover the flask with a watch crystal and place in a water bath at 20° C. for 10 minutes until the evolution of gas ceases; add 0.1 ml bromine (Br—Baker & Adamson, A.C.S.), shake and place the covered flask in a water bath at 37° C. for 20 minutes; dilute to 25 ml with distilled water and mix. Preserve in refrigerator. Stability approximately two weeks.
   - (b) Barbital buffer solution; in a 500 ml volumetric flask place 450 ml distilled water, 5.74 gm sodium diethyl barbiturate ($C_8H_{11}O_3N_2Na$—Merck, U.S.P.) and 2.08 gm aminoacetic acid ($C_2H_5O_2N$—Pfanstiehl, C.P.); shake until dissolved; adjust potentiometrically to pH 8.0 by the addition of 1 N hydrochloric acid (approximately 11.5 ml are required); dilute to volume with distilled water. Preserve in refrigerator.

Technic

Note: Due to the fact that color development varies with the temperature and the chromogenic power of different lots of reagents, absolute reliance cannot be placed on calibration curves even though variations in optical density are not large. A standard must be run simultaneously with the unknown, thus affording a convenient means by which a correction factor may be determined.

For concentrations of thiouracil in excess of 50 mg per 100 ml, dilute 2.5 ml filtrate with 2.5 ml 0.2 M p-toluenesulfonic acid (2) before proceeding with Step 5 and multiply the values obtained from the calibration table by 2.

1. In a 30 ml Erlenmeyer flask place
   6 ml distilled water
   2 ml sample from an Ostwald-Folin pipette, thoroughly rinsing out the pipette by drawing in and expelling some of the mixture several times.
2. Add 2 ml 1 M p-toluenesulfonic acid (1) while constantly agitating the flask.
3. Shake vigorously and allow to stand for 20 minutes, shaking at 5 minute intervals.
4. Filter through a 7 cm Whatman #2 paper into a dry test tube.
5. In a 10 ml graduated test tube (sample) place
   5 ml filtrate.
6. In a second 10 ml graduated test tube (reagent blank) place
   5 ml 0.2 M p-toluenesulfonic acid (2).
7. In a third 10 ml graduated test tube (standard) place
   2 ml standard thiouracil (containing 0.2 mg) (3)
   3 ml 0.2 M p-toluenesulfonic acid (2).
8. To each tube add 2.5 N sodium hydroxide (4), drop by drop, from a capillary pipette, until the pH is approximately 8.0 as determined by wide range indicator paper (5)—about 7–9 drops are usually required.
9. Add 2 ml buffered Grote reagent (6) and mix.

Note: Occasionally a slight turbidity develops after the addition of the Grote reagent—in such cases, filter through a small Schleicher and Schuell #576 paper.

10. Allow to stand at room temperature for 15 minutes—do not allow color to develop in the dark.
11. Adjust the volume in each tube to exactly 7.5 ml with distilled water and mix.
12. Measure the transmittancy of the sample against the reagent blank set at 100% transmission at a wave length of 660 mμ, obtain the value for thiouracil from the calibration table and record.
13. Measure the transmittancy of the 20 mg standard against the reagent blank set at 100% transmission at a wave length of 660 mμ and check against the value in the calibration table.
14. Calculate the concentration of thiouracil as follows.

Calculation

If the transmittancy reading obtained in Step 13 is the same as that corresponding to a concentration of 20 mg in the calibration table, no further correction need be made and the value recorded in Step 12 represents the concentration of thiouracil in mg per 100 ml sample.

If the transmittancy reading obtained in Step 13 differs from that indicated in the calibration table, the concentration of thiouracil is calculated by the following formula:

Value from calibration table (from Step 12) × $\frac{20,}{w}$

where w represents the concentration indicated by the transmittancy reading obtained in Step 13.

CALIBRATION PROCEDURE

Special Apparatus

Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.

Graduated test tubes—10 ml in 0.1 ml; the 7.5 ml graduation must be accurately calibrated.

Reagents

2. **p-Toluenesulfonic Acid, 0.2 M.** Same as for Test Procedure.
4. **Sodium Hydroxide, Volumetric 2.5 N.** Same as for Test Procedure.
5. **Wide Range Indicator Paper.** Same as for Test Procedure.
6. **Grote Reagent, Buffered (Christensen).** Same as for Test Procedure.
7. **Thiouracil Standards**
   (a) Thiouracil stock standard (1 ml contains 0.5 mg thiouracil). In a 100 ml volumetric flask place 50 mg thiouracil ($C_4H_4N_2OS$—Lederle, "Deracil"); add approximately 80 ml distilled water and shake until dissolved applying gentle heat if necessary; dilute to volume. Stable for several days in refrigerator.
   (b) Dilute standard (1 ml contains 0.1 mg thiouracil). In a 100 ml volumetric flask place 20 ml stock standard (7a) and dilute to volume with 0.2 M p-toluenesulfonic acid (2). Prepare just before use.
   (c) Calibration standards. In a series of six accurately calibrated 10 ml graduated test tubes place respectively 0, 1, 2, 3, 4 and 5 ml of the dilute standard (7b); add 5, 4, 3, 2, 1 and 0 ml 0.2 M p-toluenesulfonic acid (2) respectively, bringing the volume in each tube to 5 ml. These standards represent concentrations of thiouracil equivalent to 0, 10, 20, 30, 40 and 50 mg per 100 ml sample. Prepare just before use and proceed immediately as follows.

Technic

1. Treat each calibration standard (7c) exactly as outlined in the Test Procedure, Steps 8–11 inclusive.
2. Set the zero concentration standard at 100% transmission at a wave length of 660 mμ and record the transmittancy of the 10, 20, 30, 40 and 50 mg per cent standards. After 2 minutes repeat the reading of the 50 mg standard to make certain that the maximal value has been attained. The transmittancy of the 50 mg standard should be approximately 14%.
3. Plot the observed values on semi-log graph paper—the points fall on a straight line which passes through the origin (Calibration Curve T-15).
4. Prepare a calibration table from the T-C values obtained from the plotted curve.

Method T-15b

DETERMINATION IN URINE

SAMPLE

Aliquot of a 24-hour specimen. Keep cool during collection. Preserve with chloroform.

TEST PROCEDURE

Special Apparatus

Ostwald-Folin pipettes accurately calibrated "to contain" 1 ml.

Graduated test tubes—10 ml in 0.1 ml; the 7.5 ml graduation must be accurately calibrated.

Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.

Reagents

1. **p-Toluenesulfonic Acid, 1 M.** Same as for blood.
2. **p-Toluenesulfonic Acid, 0.2 M.** Same as for blood.
3. **Thiouracil Standard (2 ml contain 0.2 mg)** Same as for blood.
4. **Sodium Hydroxide, Volumetric 2.5 N.** Same as for blood.
5. **Wide Range Indicator Paper.** Same as for blood.
6. **Grote Reagent, Buffered (Christensen).** Same as for blood.

Technic

Note: For concentrations of thiouracil in excess of 100 mg per 100 ml, use 0.5 ml adjusted urine and 7.5 ml distilled water in Step 2 and multiply the values obtained from the calibration table for blood by 4.

1. Thoroughly mix and adjust the entire 24-hour specimen of urine to pH 8.0 with 2.5 N sodium hydroxide (4) using wide range indicator paper (5).
2. In a 30 ml Erlenmeyer flask place
   7 ml distilled water
   1 ml adjusted, unfiltered urine from an Ostwald-Folin pipette, thoroughly rinsing out the pipette by drawing in and expelling some of the mixture several times.
3. Add 2 ml 1 M p-toluenesulfonic acid (1) while constantly agitating the flask.
4. Allow to stand for 20 minutes.
5. Proceed exactly as outlined in the Test Procedure for blood, Steps 4–11 inclusive.
6. Measure the transmittancy of the sample against the reagent blank set at 100% transmission at a wave length of **660** mμ and multiply the value for thiouracil obtained from the calibration table for blood by 2 and record.
7. Measure the transmittancy of the 20 mg standard against the reagent blank set at 100% transmission at a wave length of **660** mμ and check against the value in the calibration table for blood.
8. Calculate the concentration of thiouracil as follows.

Calculation

If the transmittancy reading obtained in Step 7 is the same as that corresponding to a concentration of 20 mg in the calibration table for blood, no further correction need be made and the value recorded in Step 6 represents the concentration of thiouracil in mg per 100 ml urine.

If the transmittancy reading obtained in Step 7 differs from that indicated in the calibration table for blood, the concentration of thiouracil is calculated by the following formula:

$$\text{Value from calibration table (from Step 6)} \times \frac{20}{w},$$

where w represents the concentration indicated by the transmittancy reading obtained in Step 7.

$$\frac{\text{Volume of 24-hour specimen}}{100} \times \text{Thiouracil in mg per 100 ml} = \text{Mg thiouracil excreted in 24 hours}$$

* * *

PROCEDURE FOR USE WITH LINEAR-SCALE PHOTOELECTRIC COLORIMETERS (SHEARD-SANFORD TYPE)

TEST PROCEDURE

Exactly as outlined; set the reagent blank at 100 and use a red filter.

CALIBRATION PROCEDURE

Exactly as outlined—use a red filter; plot the observed readings on semi-log graph paper—the points fall on a straight line passing through the origin.

* * *

PROCEDURE FOR USE WITH LOGARITHMIC-SCALE PHOTOELECTRIC COLORIMETERS (KLETT-SUMMERSON TYPE)

TEST PROCEDURE

Exactly as outlined; set the reagent blank at 0 and use a red filter.

CALIBRATION PROCEDURE

Exactly as outlined—use a red filter; plot the observed readings on linear graph paper—the points fall on a straight line passing through the origin.

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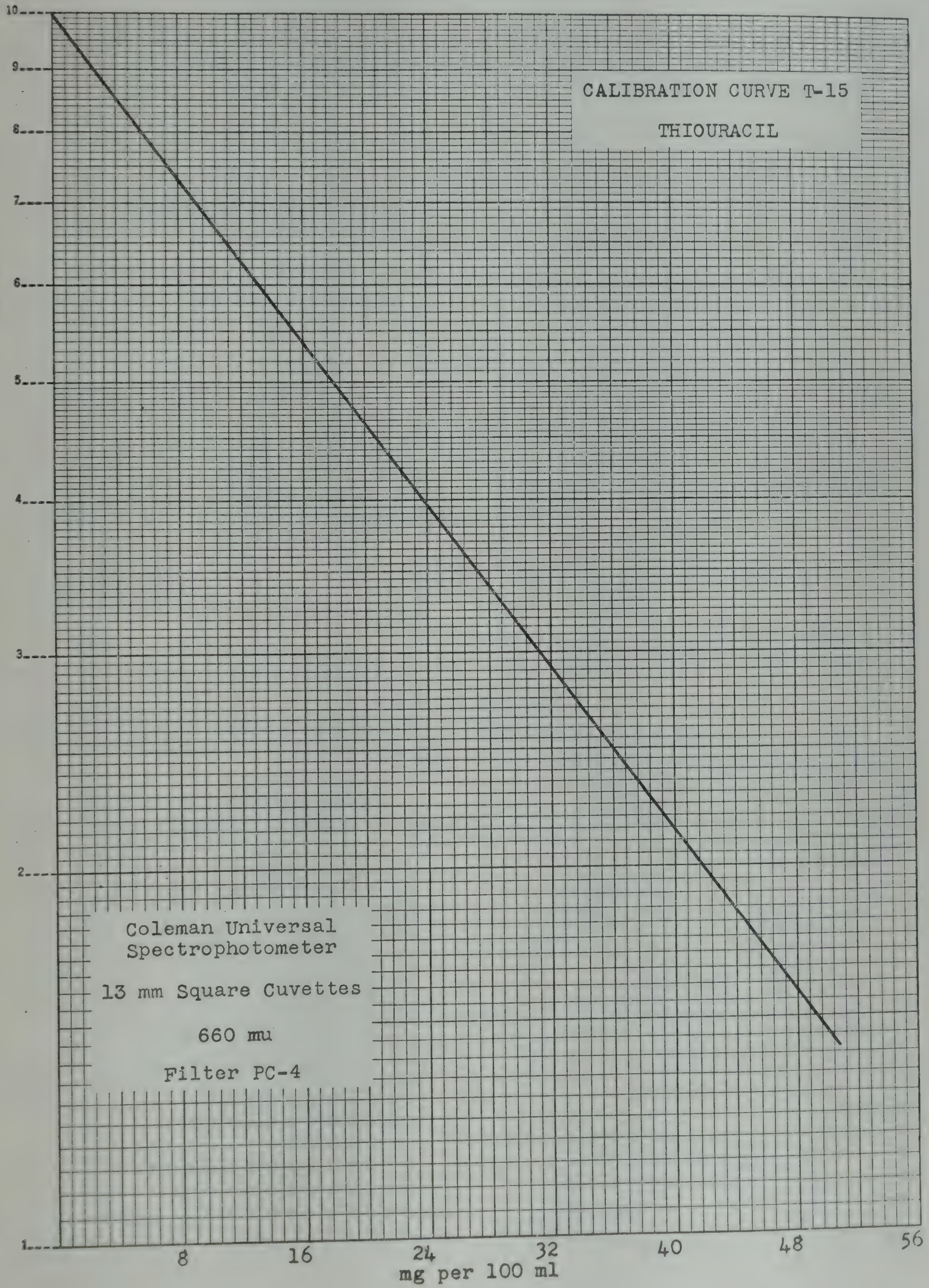
NOTES


CALIBRATION CURVE T-15
THIOURACIL
Coleman Universal Spectrophotometer
13 mm Square Cuvettes
660 mu
Filter PC-4
10
9
8
7
6
5
4
3
2
1
8
16
24
32
40
48
56
mg per 100 ml

Meter Reading	0	1	2	3	4	5	6	7	8	9
0										
10										
20										
30										
40										
50										
60										
70										
80										
90										

THYMOL TURBIDITY

A spectrophotometric adaptation based on the methods and modifications of Maclagan (1944), Shank and Hoagland (1946), Mateer, Baltz, Steele, Brouwer and Colvert (1947), Ducci (1947) and Kunkel and Hoagland (1947)

PRINCIPLE

Under certain conditions, a turbidity is formed when serum is treated with a buffered thymol solution due to the precipitation of a protein-thymol-phospholipid complex. The density of the turbidity is determined nephelometrically and expressed in terms of thymol (Maclagan) units. The turbidity produced by a 10 mg per cent protein standard or its equivalent is designated as one thymol unit.

DETERMINATION IN SERUM

SAMPLE

Serum from blood withdrawn during post-absorptive state. Although precautions should be taken to avoid hemolysis, liberated hemoglobin, unless excessive, shows negligible absorption at the wave length used. The same wave length also largely eliminates the interference caused by bilirubin.

TEST PROCEDURE

Special Apparatus

Micro pipettes, accurately calibrated "to contain" 0.1 ml.

Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.

Reagent

1. **Thymol-Barbital Buffer Solution, pH 7.55 (Mateer).** In a 1000 ml Florence flask place 1.45 gm barbituric acid ($C_4H_4O_3N_2 \cdot 2H_2O$—Merck, U.S.P.); add 1.0 gm sodium diethyl barbiturate ($C_8H_{11}O_3N_2Na$—Merck, U.S.P.); add approximately 3 gm crystalline thymol ($C_{10}H_{14}O$—Merck, Reagent); add 500 ml distilled water and heat to boiling; shake well and allow to cool to room temperature—the solution is now turbid; seed with a few small thymol crystals and shake thoroughly; allow to stand over night at room temperature; shake thoroughly and filter off the clear liquid. Adjust potentiometrically to pH 7.55 by adding either finely powdered barbituric acid or sodium diethyl barbiturate as required and checking after each addition. The solution tends to become turbid upon standing—slight turbidity clears when it is added to the serum; definitely cloudy buffers should not be used. Do not store in refrigerator—a temperature of 20° C. is preferable. Keep in tightly stoppered bottle and avoid undue exposure to air.

Technic

Note: Sera which have been preserved in the refrigerator must be allowed to come to a temperature of 21–25° C. before adding the buffered thymol.

1. In a cuvette (sample) place
   6 ml thymol-barbital buffer (1)
   0.1 ml serum from a micro pipette, thoroughly rinsing out the pipette by drawing in and expelling some of the mixture several times.
2. In another cuvette (reagent blank) place
   6 ml thymol-barbital buffer (1)
   0.1 ml distilled water.
3. Allow to stand at 25° C. for 30 minutes.
4. Mix thoroughly by inversion.
5. Measure the transmittancy of the sample against the reagent blank set at 100% transmission at a wave length of **650 mμ** and obtain the thymol unit equivalent from the calibration table.

CALIBRATION PROCEDURE

Special Apparatus

Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.

Reagents

2. **Turbidity Standards**
   - (a) Concentrated turbidity standard, Shank-Hoagland (density equivalent to 20 thymol units). In a 100 ml volumetric flask place 3 ml 0.0962 N barium chloride (i) and dilute to volume with 0.2 N sulfuric acid (ii), which has been previously cooled to 10° C. Prepare just before use.
     - (i) Barium chloride, volumetric 0.0962 N; dissolve 1.173 gm barium chloride ($BaCl_2 \cdot 2H_2O$—Baker & Adamson, A.C.S.) in distilled water and dilute to 100 ml.
     - (ii) Sulfuric acid, volumetric 0.2 N; slowly add 6 ml concentrated sulfuric acid (H_2SO_4—Grasselli, A.C.S.) to 1000 ml distilled water and mix; standardize against 0.1 N alkali using phenolphthalein as indicator—10 ml of 0.2 N acid require 20 ml of 0.1 N alkali.
   - (b) Calibration standards. In a series of five cuvettes place respectively 0, 2.5, 5.0, 7.5 and 10 ml of the concentrated turbidity standard (2a); add 10, 7.5, 5.0, 2.5 and 0 ml distilled water respectively, bringing the

volume in each cuvette to 10 ml. These standard suspensions are equivalent to approximately 0, 5, 10, 15 and 20 thymol units. Prepare just before use and proceed immediately as follows.

Technic

1. Allow the calibration standards (2b) to stand at 25° C. for 30 minutes.
2. Set the zero concentration standard at 100% transmission at a wave length of **650** mμ and record the transmittancy of the 5, 10, 15 and 20 thymol unit standards. The transmittancy of the 20 unit standard should be approximately 20%.
3. Plot the observed values on semi-log graph paper—the points fall on a straight line which passes through the origin (Calibration Curve T-20).
4. Prepare a calibration table from the T-C values obtained from the plotted curve.

Note: Whereas the calibration curves for all colorimetric procedures listed in this manual are interchangeable from one Coleman spectrophotometer to another and even from one model to another, the calibration curves for the nephelometric procedures are not interchangeable. Calibration curves must be prepared for every instrument.

A divergence of opinion as to the method of carrying out the thymol turbidity test as well as to the thymol unit values assigned to various standard calibration suspensions has resulted in considerable confusion regarding the proper technic to be employed and the interpretation of the results. The procedure outlined above seems to be the one generally in use at the present time. If 0.0962 M instead of 0.0962 N barium chloride is used in the preparation of the standard stock suspension, the values assigned to the calibration suspensions are one-half those specified in the outlined procedure.

PROCEDURE FOR USE WITH LINEAR-SCALE PHOTOELECTRIC COLORIMETERS (SHEARD-SANFORD TYPE)

TEST PROCEDURE

Exactly as outlined; set the reagent blank at 100 and use a red filter.

CALIBRATION PROCEDURE

Exactly as outlined—use a red filter; plot the observed readings on semi-log graph paper—the points fall on a straight line passing through the origin.

PROCEDURE FOR USE WITH LOGARITHMIC-SCALE PHOTOELECTRIC COLORIMETERS (KLETT-SUMMERSON TYPE)

TEST PROCEDURE

Exactly as outlined; set the reagent blank at 0 and use a red filter.

CALIBRATION PROCEDURE

Exactly as outlined—use a red filter; plot the observed readings on linear graph paper—the points fall on a straight line passing through the origin.

Note: For instruments with cells, cuvettes or tubes requiring a final volume of more than 6 ml, use double the amount of serum and thymol-barbital buffer in the test procedure. The calibration procedure is followed exactly as outlined.

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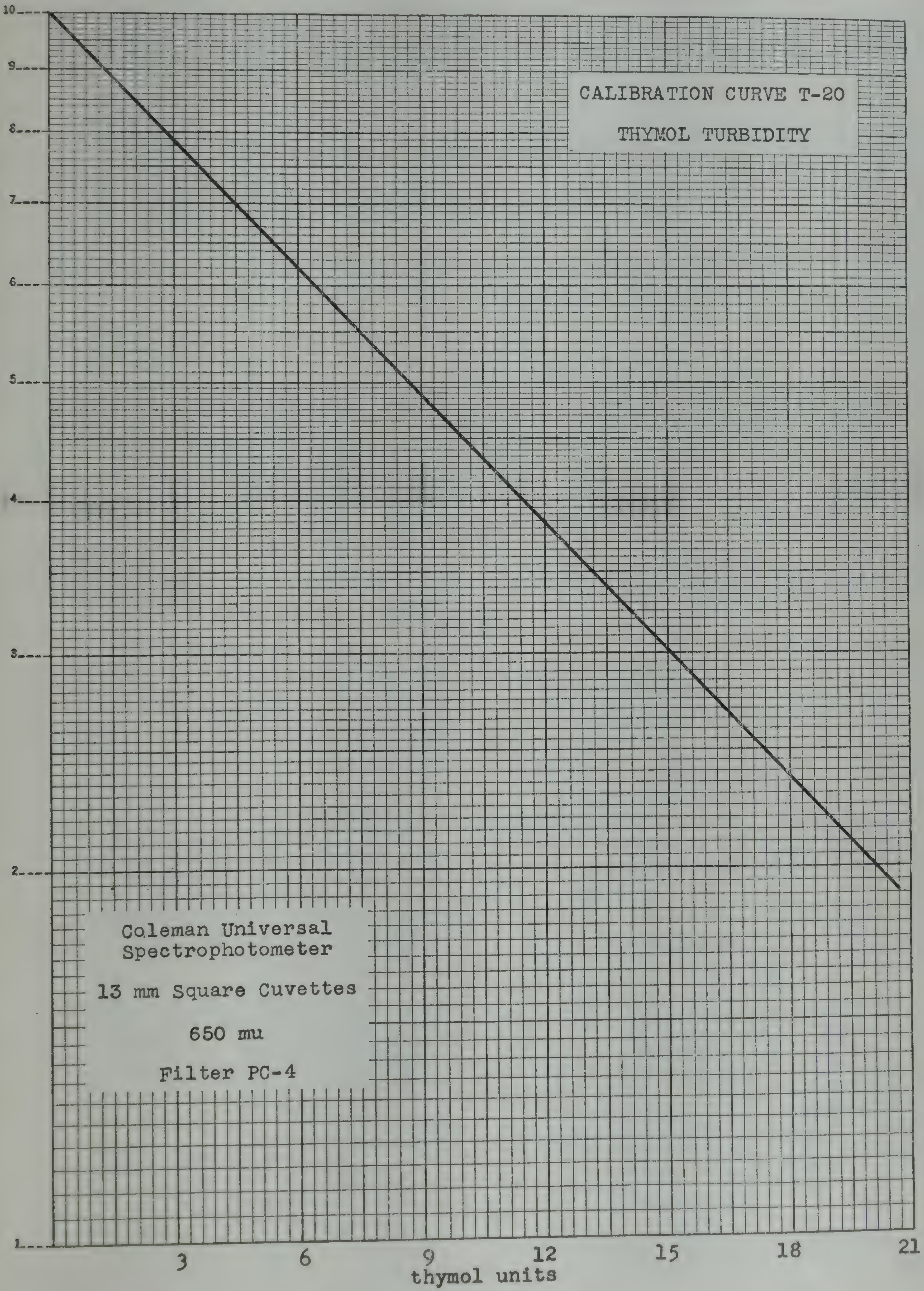
CALIBRATION CURVE T-20
THYMOL TURBIDITY
Coleman Universal Spectrophotometer
13 mm Square Cuvettes
650 mu
Filter PC-4
10
9
8
7
6
5
4
3
2
1
3
6
9
12
15
18
21
thymol units

Meter Reading	0	1	2	3	4	5	6	7	8	9
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60										
70										
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90										

UREA NITROGEN

A spectrophotometric adaptation based on the methods and modifications of Ormsby (1942) and Kawerau (1946)

PRINCIPLE

Urea, when heated with diacetyl monoxime in acid solution, forms a yellow color (Fearon reaction), which is increased in intensity by oxidation with arsenic acid.

DETERMINATION IN WHOLE BLOOD, PLASMA OR SERUM

SAMPLE

Blood withdrawn during post-absorptive state. Anticoagulant—potassium oxalate.

TEST PROCEDURE

Special Apparatus

Ostwald-Folin pipettes accurately calibrated "to contain" 1 ml.

Graduated test tubes—25 ml in 0.5 ml; the 10 ml graduation must be accurately calibrated.

Glass spheres or balls to loosely fit the mouths of the above tubes.

Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.

Reagents

1. **Sulfuric Acid, Volumetric N/12.** Slowly add 2.5 ml concentrated sulfuric acid (H_2SO_4—Grasselli, A.C.S.) to 1000 ml distilled water and mix well; titrate against standard 0.1 N alkali using phenolphthalein as indicator; adjust accordingly and re-titrate—20 ml of N/12 acid require 16.7 ml of 0.1 N alkali.
2. **Sodium Tungstate, 10% Aqueous.** Dissolve 10 gm sodium tungstate ($Na_2WO_4 \cdot 2H_2O$—Mallinckrodt, Folin Special) in distilled water and dilute to 100 ml. This solution must be neutral or slightly alkaline to phenolphthalein, not more than 0.4 ml being required to neutralize 10 ml of 0.1 N hydrochloric acid. Preserve in resistant-glass bottle.
3. **Diacetyl Monoxime Reagent (Kawerau).** Dissolve 1 gm diacetyl monoxime ($C_4H_7O_2N$—Eastman #86) in 100 ml 5% acetic acid (a); decolorize with Norit A (Pfanstiehl) if necessary. Discard when a discoloration or precipitate appears.
   (a) Acetic acid, 5% aqueous; dilute 5 ml glacial acetic acid (CH_3COOH—Baker & Adamson, A.C.S.) to 100 ml with distilled water.
4. **Arsenic-Hydrochloric Acid Oxidizing Mixture (Kawerau).** Dissolve 10 gm crystalline arsenic acid (H_3AsO_4—Baker & Adamson, Reagent) in 100 ml concentrated hydrochloric acid (HCl—Baker & Adamson, A.C.S.); allow to stand until clear. Use clear, pale yellow supernatant fluid. Preserve in glass-stoppered bottle.

Technic

Note: For concentrations of urea nitrogen less than 10 mg per 100 ml, omit Step 4 and divide the values obtained from the calibration table by 2; for concentrations in excess of 60 mg per 100 ml, use 1 ml filtrate and 1 ml distilled water in Step 6 and multiply the values obtained from the calibration table by 2.

1. In a 25 ml Erlenmeyer flask place
   8 ml N/12 sulfuric acid (1)
   1 ml sample from an Ostwald-Folin pipette, thoroughly rinsing out the pipette by drawing in and expelling some of the mixture several times.
2. Mix the contents of the flask by gentle rotation until the fluid is uniformly black-brown.
3. Add 1 ml 10% sodium tungstate (2), drop by drop, while constantly but gently rotating the flask; shake the flask vigorously until the mixture is chocolate colored and foam-free.
4. Add 10 ml distilled water and mix thoroughly.
5. Filter through a dry 11 cm Whatman #4 paper into a dry test tube.
6. In a 25 ml graduated test tube (sample) place
   2 ml filtrate.
7. In another 25 ml graduated test tube (reagent blank) place
   2 ml distilled water.
8. To each tube add
   2 ml diacetyl monoxime reagent (3) and mix
   3 ml arsenic-hydrochloric oxidant (4) and mix
   2 ml distilled water and mix.
9. Place in boiling water for 20 minutes, closing the mouths of the tubes loosely with glass spheres; the level of the fluid in the tubes must be below the level of the water in the bath.
10. Cool under running tap water for 3 minutes.
11. Adjust the volume to 10 ml with distilled water.
12. Measure the transmittancy of the sample against the reagent blank set at 100% transmission at a wave length of 475 mμ and obtain the concentration of urea nitrogen from the calibration table.

CALIBRATION PROCEDURE

Special Apparatus

Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.

Graduated test tubes—25 ml in 0.5 ml; the 10 ml graduation must be accurately calibrated.

Glass spheres or balls to loosely fit the mouths of the above tubes.

Reagents

3. **Diacetyl Monoxime Reagent (Kawerau).** Same as for Test Procedure.
4. **Arsenic-Hydrochloric Acid Oxidizing Mixture (Kawerau).** Same as for Test Procedure.
5. **Urea Nitrogen Standards**
    - (a) Urea nitrogen stock standard, Karr (1 ml contains 0.3 mg urea nitrogen). Dry recrystallized urea ($(NH_2)_2CO$—Reagent Chemicals, Special) at 100° C. for one hour and cool in desiccator to constant weight; transfer 64.3 mg to a 100 ml volumetric flask; add approximately 50 ml distilled water, shake until dissolved and dilute to volume. Preserve in refrigerator.
    - (b) Dilute standard (1 ml contains 0.03 mg urea nitrogen). In a 100 ml volumetric flask place 10 ml stock standard (5a) and dilute to volume with distilled water. Prepare just before use.
    - (c) Calibration standards. In a series of five accurately calibrated 25 ml graduated test tubes place respectively 0, 0.5, 1.0, 1.5 and 2.0 ml of the dilute standard (5b); add 2.0, 1.5, 1.0, 0.5 and 0 ml distilled water respectively, bringing the volume in each tube to 2 ml. These standards represent concentrations of urea nitrogen equivalent to 0, 15, 30, 45 and 60 mg per 100 ml sample. Prepare just before use.

Technic

1. Treat each calibration standard (5c) exactly as outlined in the Test Procedure, Steps 8–11 inclusive.
2. Set the zero concentration standard at 100% transmission at a wave length of 475 mμ and record the transmittancy of the 15, 30, 45 and 60 mg per cent standards. The transmittancy of the 60 mg standard should be approximately 15.5%.
3. Plot the observed values on semi-log graph paper —the points fall on a straight line which passes through the concentration axis to the right of the origin (Calibration Curve U-10).
4. Prepare a calibration table from the T-C values obtained from the plotted curve.

CONVERSION FACTOR

Urea nitrogen in mg per 100 ml × 2.14 = Urea (as such) in mg per 100 ml

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Method U-10b

DETERMINATION IN URINE

SAMPLE

Aliquot of a 24-hour specimen. Keep cold during collection. Since ammonia causes no interference it need not be removed from the urine before analysis.

TEST PROCEDURE

Special Apparatus

As listed under the Test Procedure for blood.

Reagents

1. **Sulfuric Acid, Volumetric N/12.** Same as for blood.
2. **Sodium Tungstate, 10% Aqueous.** Same as for blood.
3. **Diacetyl Monoxime Reagent (Kawerau).** Same as for blood.
4. **Arsenic-Hydrochloric Acid Oxidizing Mixture (Kawerau).** Same as for blood.

Technic

Note: For concentrations of urea nitrogen less than 200 mg per 100 ml, use 10 ml urine in Step 2 and multiply the values obtained from the calibration table for blood by 10; for concentrations in excess of 1200 mg per 100 ml, use 2.5 ml urine in Step 2 and multiply the values obtained from the calibration table by 40.

1. Filter a small portion of thoroughly mixed urine.
2. In a 100 ml volumetric flask place 5 ml filtered urine and dilute to volume with distilled water.
3. In a 25 ml Erlenmeyer flask place
    - 8 ml N/12 sulfuric acid (1)
    - 1 ml diluted urine from an Ostwald-Folin pipette, thoroughly rinsing out the pipette by drawing in and expelling some of the mixture several times.
4. Proceed exactly as outlined in the Test Procedure for blood, Steps 2–11 inclusive.
5. Measure the transmittancy of the sample against the reagent blank set at 100% transmission at a wave length of 475 mμ and multiply the value from the calibration table for blood by 20 to obtain the concentration of urea nitrogen in mg per 100 ml urine.

Calculation

$$\frac{\text{Urea nitrogen in mg per 100 ml}}{1000} \times \frac{\text{Volume 24-hour sample}}{100} \times 2.14 = \text{Urea in gm per 24 hours}$$

PROCEDURE FOR USE WITH LINEAR-SCALE PHOTOELECTRIC COLORIMETERS (SHEARD-SANFORD TYPE)

TEST PROCEDURE

Exactly as outlined; set the reagent blank at 100 and use a blue filter.

CALIBRATION PROCEDURE

Exactly as outlined—use a blue filter; plot the observed readings on semi-log graph paper—the points fall on a smooth curve passing through the concentration axis slightly to the right of the origin.

PROCEDURE FOR USE WITH LOGARITHMIC-SCALE PHOTOELECTRIC COLORIMETERS (KLETT-SUMMERSON TYPE)

TEST PROCEDURE

Exactly as outlined; set the reagent blank at 0 and use a blue filter.

CALIBRATION PROCEDURE

Exactly as outlined—use a blue filter; plot the observed readings on linear graph paper—the points fall on a smooth curve passing through the concentration axis slightly to the right of the origin.

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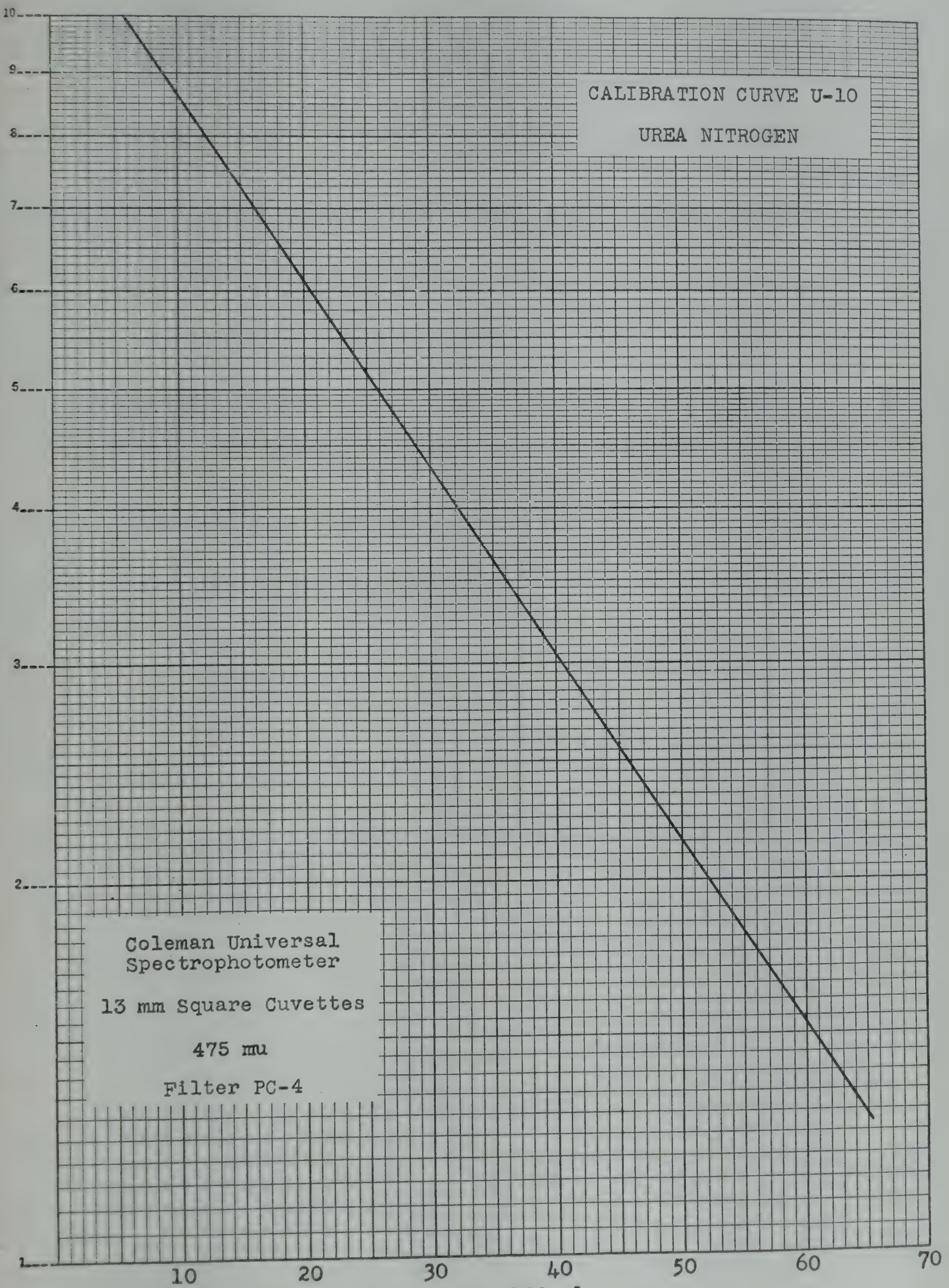
NOTES

NOTES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UREA NITROGEN
Coleman Universal Spectrophotometer
13 mm Square Cuvettes
475 mμ
Filter PC-4
10
9
8
7
6
5
4
3
2
1
10
20
30
40
50
60
70
mg per 100 ml

Meter Reading	0	1	2	3	4	5	6	7	8	9
0										
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UREA NITROGEN

A spectrophotometric adaptation based on the methods and modifications of Folin and Bell (1917), Folin and Wu (1919), Karr (1924), Koch (1926) and Gentzkow and Masen (1942)

PRINCIPLE

The enzyme urease, at a definite pH, quantitatively hydrolyzes urea into ammonium carbonate. The ammonia is then determined by means of the reddish-orange color developed with alkaline potassium mercuric iodide (Nessler reaction), stabilized with potassium gluconate and potassium persulfate.

DETERMINATION IN WHOLE BLOOD, PLASMA OR SERUM

SAMPLE

Blood withdrawn during post-absorptive state. Anticoagulant—potassium oxalate.

TEST PROCEDURE

Special Apparatus

Ostwald-Folin pipettes accurately calibrated "to contain" 1 ml.

Graduated test tubes—25 ml in 0.5 ml; the 15 ml graduation must be accurately calibrated. Note: These tubes must be thoroughly cleaned after use with dilute nitric acid to remove all traces of mercury in order to prevent inhibition of the action of the enzyme.

Capillary droppers delivering 40 drops per ml.

Thin glass rods, one end bent so as to form a small sharp hook.

Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.

Reagents

1. **Water, Distilled, Ammonia-Free.** In an all-glass Pyrex distillation apparatus place 1 gm sodium carbonate (Na_2CO_3—Baker & Adamson, A.C.S.) and 1 gm potassium permanganate ($KMnO_4$—Baker & Adamson, A.C.S.); add 500 ml distilled water; redistil, rejecting the first 100 ml portion and collecting only the next 300 ml. Use for the preparation of all reagents and standards and where indicated in the test procedure.
2. **Sulfuric Acid, Volumetric N/12.** Add 2.5 ml concentrated sulfuric acid (H_2SO_4—Grasselli, A.C.S.) to 1000 ml ammonia-free distilled water (1) and mix well; titrate against standard 0.1 N alkali using phenolphthalein as indicator; adjust accordingly and re-titrate—20 ml of N/12 acid require 16.7 ml of 0.1 N alkali.
3. **Sodium Tungstate, 10% Aqueous.** Dissolve 10 gm sodium tungstate ($Na_2WO_4 \cdot 2H_2O$—Mallinckrodt, Folin Special) in ammonia-free distilled water (1) and dilute to 100 ml. The solution must be neutral or slightly alkaline to phenolphthalein, not more than 0.4 ml being required to neutralize 10 ml 0.1 N hydrochloric acid. Preserve in resistant-glass bottle.
4. **Urease Paper (Folin).** In a clean 250 ml Florence flask place 30 gm jack bean meal (Arlington) and 100 ml 28.5% ethyl alcohol (a); add 1 ml acetate buffer solution (Reagent #6); stopper with a clean cork and shake vigorously for 5 minutes and then more slowly for 10 additional minutes; filter, or centrifuge for one-half hour in small portions covering the mouths of the tubes with tinfoil; transfer the filtrate or centrifugates to a Petri dish and immediately take up on one inch strips of Whatman #1 filter paper; hang up the strips on threads in such a manner that the dependent portions of the strips do not cling together; allow to dry in the absence of air currents; when thoroughly dry, cut the strips transversely in $^1/_4$ inch pieces thus giving strips approximately 1×2.5 cm in size. Preserve in a dry, wide-mouth bottle. Paper impregnated with urease in this manner retains its activity for years.
    (a) Ethyl alcohol, 28.5% aqueous; dilute 30 ml 95% ethyl alcohol (C_2H_5OH—U. S. Industrial Chemicals, U.S.P.) to 100 ml with ammonia-free distilled water (1).

Note: Instead of urease paper, a glycerine extract of urease may be used; however, an appreciable ammonia blank may develop even when comparatively fresh.

5. **Urease-Glycerol Extract (Koch).** In a clean 250 ml Florence flask place 15 gm permutit (Arlington, According to Folin); add 200 ml 2% acetic acid (a); shake thoroughly, allow to settle, decant and discard the supernatant fluid; wash the permutit with three 200 ml portions of distilled water, shaking, decanting and discarding the wash fluid as before; to the moist permutit add 50 ml 0.001 N sulfuric acid (b) and 30 gm jack bean meal (Arlington); shake gently for 30 minutes; add 150 ml glycerine ($C_3H_5(OH)_3$—Merck, A.C.S.) and mix thoroughly; allow to stand over night in refrigerator; filter through several thicknesses of gauze; centrifuge the filtrate until the supernatant fluid shows only a slight opalescence. Preserve in refrigerator. Check the blank value frequently. Stability about one year.
    (a) Acetic acid, 2% aqueous; dilute 2 ml glacial acetic acid (CH_3COOH—Baker & Adamson, A.C.S.) to 100 ml with distilled water.
    (b) Sulfuric acid, volumetric 0.001 N; dilute 1.2 ml N/12 sulfuric acid (2) to 100 ml with distilled water.

6. **Acetate Buffer Solution (Karr).** Dissolve 20 gm crystalline sodium acetate ($NaC_2H_3O_2 \cdot 3H_2O$—Baker & Adamson, A.C.S.) in 80 ml ammonia-free distilled water (1); add 2.2 ml 10% acetic acid (a); dilute to 100 ml. Preserve with a few drops of chloroform.
   - (a) Acetic acid, 10% aqueous; dilute 10 ml glacial acetic acid (CH_3COOH—Baker & Adamson, A.C.S.) to 100 ml with ammonia-free distilled water (1).
7. **Nesslerizing Solution (Gentzkow-Masen).** Mix 2.5 ml 1% potassium gluconate (a) and 2.5 ml one-third saturated potassium persulfate (b) and pour the mixture into 5 ml Gentzkow-Masen nessler reagent (c); mix and use within 15 minutes.
   - (a) Potassium gluconate, 1% aqueous; dissolve 1 gm potassium gluconate ($KC_7H_{13}O_8$—Mallinckrodt) in ammonia-free distilled water (1) and dilute to 100 ml. Preserve in refrigerator. Prepare fresh weekly.
   - (b) Potassium persulfate, one-third saturated; dilute 10 ml saturated potassium persulfate (i) to 30 ml with ammonia-free distilled water (1). Preserve in refrigerator. Prepare fresh weekly.
     - (i) Potassium persulfate, saturated aqueous; place approximately 8 gm potassium persulfate ($K_2S_2O_8$—Merck, Low-Nitrogen Special) in a brown glass bottle; add 100 ml ammonia-free distilled water (1) and shake thoroughly. A portion of the salt remains undissolved and settles to the bottom—do not remove the undissolved persulfate; an excess must always be present to compensate for decomposition. Shake shortly before use; allow to settle and use the clear supernatant fluid. Preserve in refrigerator.
   - (c) Nessler reagent (Gentzkow-Masen); in a 1000 ml volumetric flask place 150 ml Folin-Wu double iodide solution (i), 500 ml 2.5 N sodium hydroxide (ii) and dilute to volume with ammonia-free distilled water (1); allow to stand for three days to permit sedimentation of the small amount of precipitate which forms. Preserve in ceresin-lined bottle. Stability indefinite. Note: The alkaline concentration of this reagent is 5% instead of 7% as in the usual Folin-Wu reagent—15.5—16.0 ml must be required to neutralize 20 ml 1 N hydrochloric acid, using phenolphthalein as indicator.
     - (i) Double iodide solution (Folin-Wu); in a 500 ml Florence flask place 150 gm granular potassium iodide (KI—Mallinckrodt, A.C.S.) and 100 gm resublimed iodine (I—Baker & Adamson, A.C.S.); add 100 ml ammonia-free distilled water (1) and shake until the iodine has completely dissolved; add 150 gm redistilled metallic mercury (Hg—Baker & Adamson, A.C.S.); shake the flask continuously and vigorously until the red color of the iodine solution has become visibly pale, though still red; immediately start cooling under running tap water, continuing vigorous shaking, until the reddish color has been replaced by the greenish-yellow color of the double iodide. If properly prepared, the solution is clear and there is a residue of shining metallic mercury; solutions showing a gray-green sludge are unsatisfactory. The whole operation should consume no more than 10–15 minutes. Since the reaction develops considerable heat, precautions should be taken to protect the hands during the shaking procedure. Decant the solution into a 2000 ml mixing cylinder, washing mercury and flask with liberal quantities of ammonia-free distilled water and decanting the washings into the cylinder. Dilute to volume with ammonia-free distilled water.
     - (ii) Sodium hydroxide, volumetric 2.5 N; prepare by diluting slightly more than the required amount of concentrated carbonate-free sodium hydroxide solution (see Appendix); dilute 20 ml of this approximately 2.5 N solution to 100 ml; titrate against standard 0.5 N acid using phenolphthalein as indicator; adjust accordingly and re-titrate—20 ml of 0.5 N acid require 20 ml of a 1:5 dilution of 2.5 N alkali. Preserve in ceresin-lined bottle.

Technic

Note: For concentrations of urea nitrogen in excess of 60 mg per 100 ml, dilute one volume of the protein-free filtrate in Step 7 with two or three volumes of ammonia-free distilled water (1) and multiply the values obtained from the calibration table by 3 or 4 respectively.

1. In a 30 ml Erlenmeyer flask place
   8 ml N/12 sulfuric acid (2).
2. Add 1 ml sample from an Ostwald-Folin pipette, thoroughly rinsing out the pipette by drawing in and expelling some of the mixture several times.
3. Mix the contents of the flask by gentle rotation until the fluid is uniformly black-brown.
4. Add 1 ml 10% sodium tungstate (3), drop by drop, while constantly but gently rotating the flask.

5. Shake the flask vigorously until the mixture is chocolate brown and foam-free.
6. Filter through a dry 9 cm Whatman #4 paper into a dry test tube (double acid-washed quantitative filter paper must not be used).
7. After filtration is nearly complete pour the filtrate, even though clear, back on the filter paper and refilter.
8. In each of two 25 ml graduated test tubes place 1 loosely coiled strip urease paper (4) or 2 drops urease-glycerol extract (5).
9. To one tube (sample) add
   4 ml ammonia-free distilled water (1)
   1 ml protein-free filtrate.
10. To the other tube (reagent blank) add 5 ml ammonia-free distilled water (1).
11. To each tube add 0.5 ml acetate buffer solution (6).
12. Place tubes in water bath at 45° C. for 15 minutes, shaking thoroughly at 5 minute intervals (the shaking is absolutely essential when urease paper is used).
13. Remove tubes from water bath and with a hooked glass rod slide the strips of urease paper up the sides of the tubes until about one-half inch from the top.
14. Add 5 ml ammonia-free distilled water (1), allowing the water to fall on the papers and flow down the sides of the tubes; remove papers.
15. Place tubes in a beaker half-full of ice-cold water.

Note: Carry out Steps 15–18 inclusive without removing the tubes from the cold water bath except when adjusting volumes.

16. After 10 minutes in the cold water bath, dilute to 11 ml with ammonia-free distilled water (1) and mix.
17. Add 4 ml freshly prepared nesslerizing solution (7), allowing the solution to fall directly into the fluid in the tube, and mix by lateral shaking.
18. Adjust the volume, if necessary, to exactly 15 ml with ammonia-free distilled water (1).
19. Remove tubes from water bath and allow to stand at room temperature (25° C.) for 15 minutes.
20. Measure the transmittancy of the sample against the reagent blank set at 100% transmission at a wave length of 500 mμ and obtain the concentration of urea nitrogen from the calibration table.

CALIBRATION PROCEDURE

Special Apparatus

Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.

Graduated test tubes—25 ml in 0.5 ml; the 15 ml graduation must be accurately calibrated. Note: These tubes must be thoroughly cleaned after use with dilute nitric acid to remove all traces of mercury in order to prevent inhibition of the action of the enzyme.

Capillary droppers delivering 40 drops per ml.

Thin glass rods, one end bent so as to form a small sharp hook.

Reagents

1. **Water, Distilled, Ammonia-Free.** Same as for Test Procedure.
4. **Urease Paper (Folin).** Same as for Test Procedure.

   or

5. **Urease-Glycerol Extract (Koch).** Same as for Test Procedure.
6. **Acetate Buffer Solution (Karr).** Same as for Test Procedure.
7. **Nesslerizing Solution (Gentzkow-Masen).** Same as for Test Procedure.
8. **Urea Nitrogen Standards**
   (a) Urea nitrogen stock standard, Karr (1 ml contains 0.3 mg urea nitrogen). Dry recrystallized urea ($((NH_2)_2)CO$—Reagent Chemicals, Special) at 100° C. for one hour and cool in desiccator to constant weight. Transfer 64.3 mg to a 100 ml volumetric flask; add approximately 50 ml ammonia-free distilled water (1); shake until dissolved and dilute to volume with same.
   (b) Dilute standard (1 ml contains 0.015 mg urea nitrogen). In a 100 ml volumetric flask place 5 ml stock standard (8a) and dilute to volume with ammonia-free distilled (1). Prepare just before use.
   (c) Calibration standards. In a series of five accurately calibrated 25 ml graduated test tubes place respectively 0, 1, 2, 3 and 4 ml of the dilute standard (8b); add 5, 4, 3, 2 and 1 ml ammonia-free distilled water (1) respectively, bringing the volume in each tube to 5 ml. These standards represent concentrations of urea nitrogen equivalent to 0, 15, 30, 45 and 60 mg per 100 ml sample. Prepare just before use and proceed immediately as follows.

Technic

Note: The calibration procedure must be carried out with the form of enzyme to be employed in the test procedure—the calibration must not be made with urease paper if the urease extract is to be used in the determination, and vice versa.

1. In each calibration standard (8c) place 1 loosely coiled strip urease paper (4) or 2 drops urease-glycerol extract (5).
2. Proceed exactly as outlined in the Test Procedure, Steps 11–19 inclusive.
3. Set the zero concentration standard at 100% transmission at a wave length of 500 mμ and record

the transmittancy of the 15, 30, 45 and 60 mg per cent standards. The transmittancy of the 60 mg standard should be approximately 41%.

4. Plot the observed values on semi-log graph paper—the points fall on a very slight curve which passes through the concentration axis slightly to the right of the origin (Calibration Curve U-11).
5. Prepare a calibration table from the T-C values obtained from the plotted curve.

CONVERSION FACTOR

Urea nitrogen in mg per 100 ml × 2.14 = Urea (as such) in mg per 100 ml

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Method U-11b

DETERMINATION IN URINE

SAMPLE

Aliquot of a 24-hour specimen. Keep cold during collection. Ammonia must be removed by treatment with permutit.

TEST PROCEDURE

Special Apparatus

As listed under the Test Procedure for blood.

Reagents

1. **Water, Distilled, Ammonia-Free.** Same as for blood.
2. **Sulfuric Acid, Volumetric N/12.** Same as for blood.
3. **Sodium Tungstate, 10% Aqueous.** Same as for blood.
4. **Urease Paper (Folin).** Same as for blood.

or

5. **Urease-Glycerol Extract (Koch).** Same as for blood.
6. **Acetate Buffer Solution (Karr).** Same as for blood.
7. **Nesslerizing Solution (Gentzkow-Masen).** Same as for blood.
9. **Permutit (Arlington, According to Folin).** Use the 60–80 mesh material. A suspension in water must settle quickly and leave a clear supernatant fluid. It is best to wash each new lot before use by shaking with an excess of 2% acetic acid, decanting and discarding the wash fluid and repeating this process using ammonia-free distilled water (1) instead of acetic acid until the supernatant fluid is clear—at least three washings with water should be made after the acid washing. Used permutit may be regenerated in the same manner.

Technic

Note: For concentrations of urea nitrogen less than 150 mg per 100 ml, use 20 ml urine in Step 2 and multiply the values obtained from the calibration table for blood by 10; for concentrations in excess of 1200 mg per 100 ml, use 5 ml urine in Step 2 and multiply the values obtained from the calibration table by 40.

1. Filter a small portion of thoroughly mixed urine.
2. In a 50 ml Erlenmeyer flask place
   5 ml ammonia-free distilled water (1)
   10 ml filtered urine from an Ostwald-Folin pipette, thoroughly rinsing out the pipette by drawing in and expelling some of the mixture several times.
3. Add 5 gm permutit (9).
4. Gently shake the flask in a rotatory manner for 5 minutes continuously.
5. Add 25 ml ammonia-free distilled water (1), mix and allow the permutit to settle.
6. Decant the supernatant fluid through a dry Whatman #4 filter paper into a 200 ml volumetric flask.
7. Repeat Steps 5 and 6 twice.
8. Dilute to volume with ammonia-free distilled (1) and mix.
9. Proceed exactly as outlined in the Test Procedure for blood, Steps 1–19 inclusive.
10. Measure the transmittancy of the sample against the reagent blank set at 100% transmission at a wave length of **500 mμ** and multiply the value from the calibration table for blood by 20 to obtain the concentration of urea nitrogen in mg per 100 ml urine.

Calculation

$$\frac{\text{Urea nitrogen in mg per 100 ml}}{1000} \times \frac{\text{Volume 24-hour sample}}{100} \times 2.14 = \text{Urea in gm per 24 hours}$$

PROCEDURE FOR USE WITH LINEAR-SCALE PHOTOELECTRIC COLORIMETERS (SHEARD-SANFORD TYPE)

TEST PROCEDURE

Exactly as outlined; set the reagent blank at 100 and use a blue filter.

CALIBRATION PROCEDURE

Exactly as outlined—use a blue filter; plot the observed readings on semi-log graph paper—the points fall on a straight line passing through the origin.

PROCEDURE FOR USE WITH LOGARITHMIC-SCALE PHOTOELECTRIC COLORIMETERS (KLETT-SUMMERSON TYPE)

TEST PROCEDURE

As outlined, except that the final dilution is made to 20 or 25 ml; set the reagent blank at 0 and use a blue filter.

CALIBRATION PROCEDURE

As outlined, except that the final dilution is made to 20 or 25 ml—use a blue filter; plot the observed readings on linear graph paper—the points fall on a straight line passing through the origin.

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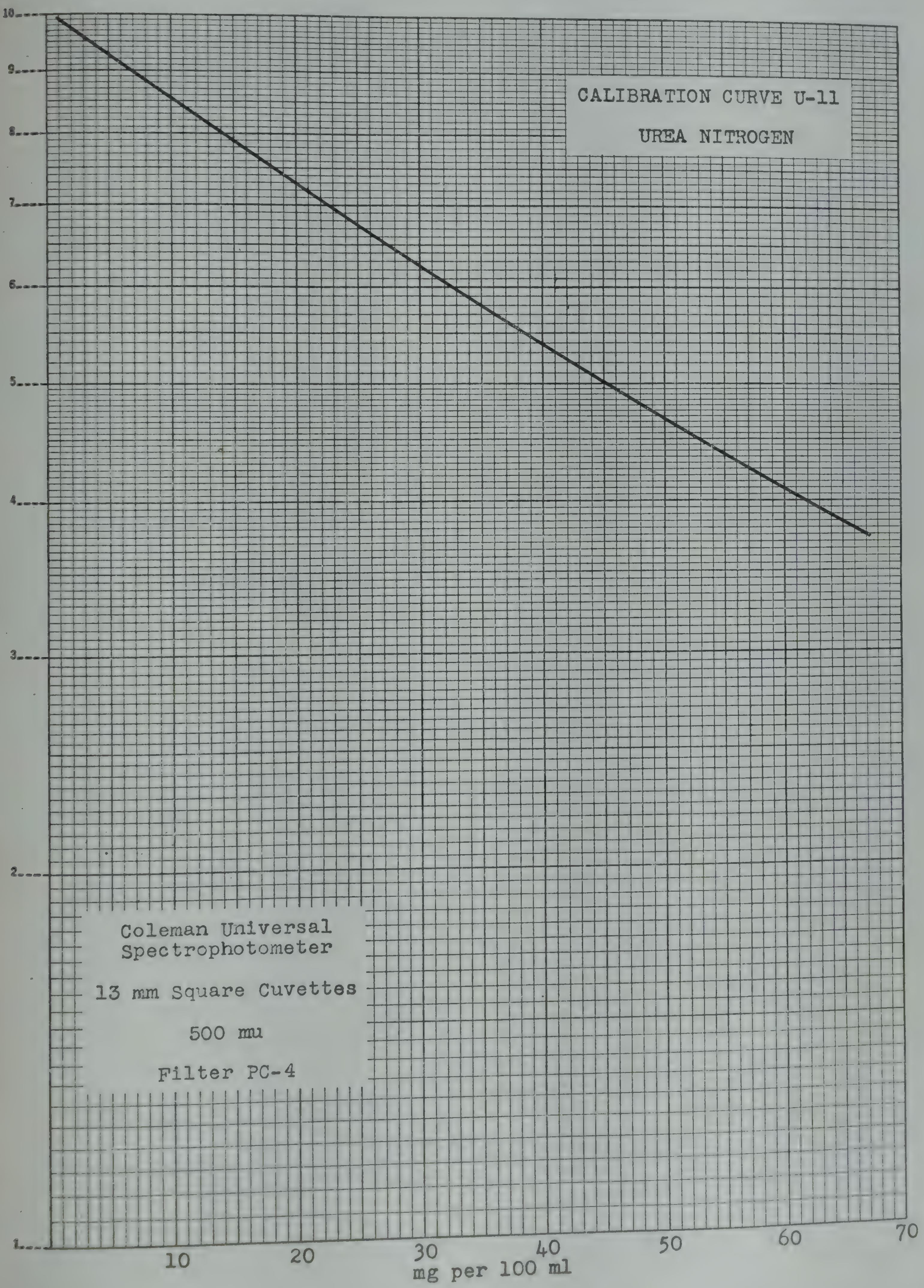
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UREA NITROGEN
Coleman Universal Spectrophotometer
13 mm Square Cuvettes
500 mu
Filter PC-4
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9
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5
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mg per 100 ml

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70										
80										
90										

URIC ACID

A spectrophotometric adaptation based on the methods and modifications of Benedict and Hitchcock (1914), Folin and Wu (1919), Benedict and Franke (1922), Folin (1930; 1933; 1934), Benedict and Behre (1931), Newton (1937) and Brown (1945)

PRINCIPLE

Uric acid, in a tungstic acid protein-free filtrate, is treated with phosphotungstic acid in the presence of cyanide and urea to produce a blue color.

DETERMINATION IN PLASMA OR SERUM

SAMPLE

Plasma or serum from blood withdrawn during postabsorptive state. Anticoagulant for plasma—lithium oxalate; avoid an excess.

TEST PROCEDURE

Special Apparatus

Ostwald-Folin pipettes accurately calibrated "to contain" 1 ml.

Graduated test tubes—25 ml in 0.5 ml; the 15 ml graduation must be accurately calibrated.

Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.

Reagents

1. **Sulfuric Acid, Volumetric N/12.** Add 2.5 ml concentrated sulfuric acid (H_2SO_4—Grasselli, A.C.S.) to 1000 ml distilled water and mix well; titrate against standard 0.1 N alkali using phenolphthalein as indicator; adjust accordingly and retitrate—20 ml of N/12 acid require 16.7 ml of 0.1 N alkali.
2. **Sodium Tungstate, 10% Aqueous.** Dissolve 10 gm sodium tungstate ($Na_2WO_4 \cdot 2H_2O$—Mallinckrodt, Folin Special) in distilled water and dilute to 100 ml. The solution must be neutral or slightly alkaline to phenolphthalein, not more than 0.4 ml being required to neutralize 10 ml 0.1 N hydrochloric acid. Preserve in resistant-glass bottle.
3. **Cyanide-Urea Reagent (Newton).** Just before use dissolve 1 gm sodium cyanide (NaCN—Merck, A.C.S.) in 20 ml 20% urea (a). Discard any excess.
    (a) Urea, 20% aqueous; dissolve 20 gm urea ($(NH_2)_2CO$—Merck, Reagent) in distilled water and dilute to 100 ml. If the solution is not crystal clear, allow to stand for several days and filter through a Schleicher and Schuell #576 paper.
4. **Phosphotungstic Acid Reagent (Brown).** In a 1000 ml Florence flask place 100 gm sodium tungstate ($Na_2WO_4 \cdot 2H_2O$—Mallinckrodt, Folin Special), 20 gm anhydrous dibasic sodium phosphate (Na_2HPO_4—Mallinckrodt, AR) and 150 ml distilled water; heat until solution is complete; slowly and with constant shaking add 100 ml warm 25% sulfuric acid (a); reflux gently for one hour; cool under running tap water to room temperature; transfer to a 1000 ml volumetric flask, thoroughly rinsing out the original flask with several portions of distilled water and transferring the washings to the volumetric flask; dilute to volume with distilled water and mix by inversion. Stable indefinitely at room temperature.
    (a) Sulfuric acid, 25% aqueous; slowly add 25 ml concentrated sulfuric acid (H_2SO_4—Grasselli, A.C.S.) to 75 ml distilled water and mix well. This should be prepared shortly before use and added, as indicated above, while still warm.

Technic

Note: Due to the variation in chromogenic power exhibited by different lots of reagents (especially the cyanide) and even by the same lot of reagents at different times, it is necessary to always run a standard simultaneously with the unknown, thus affording a convenient means by which a correction factor may be determined. Although the standard may be of any concentration, one containing 0.01 mg per ml and equivalent to 4 mg per 100 ml is recommended (for preparation see calibration standards (8c)).

For concentrations of uric acid in excess of 10 mg per 100 ml, dilute 2 ml protein-free filtrate with an equal volume of distilled water before proceeding with Step 6 and multiply the values obtained from the calibration table by 2.

1. In a 30 ml Erlenmeyer flask place
    8 ml N/12 sulfuric acid (1)
    1 ml sample from an Ostwald-Folin pipette, thoroughly rinsing outthe pipette by drawing in and expelling some of the mixture several times.
2. Mix the contents of the flask by gentle rotation until the fluid is uniformly black-brown.
3. Add 1 ml 10% sodium tungstate (2), drop by drop, while constantly but gently, rotating the flask.
4. Shake the flask vigorously until the mixture is chocolate colored and foam-free.
5. Filter through a dry 9 cm Whatman #4 paper into a dry test tube.
6. In a 25 ml graduated test tube (sample) place
    2.5 ml protein-free filtrate
    2.5 ml distilled water and mix.
7. In a second 25 ml graduated test tube (reagent blank) place
    5 ml distilled water.

8. In a third 25 ml graduated test tube (standard) place
   1 ml standard uric acid solution (containing 0.01 mg)
   4 ml distilled water.
9. To each tube add FROM A BURETTE 3 ml freshly prepared cyanide-urea reagent (3) and mix.
10. Add 1 ml phosphotungstic acid reagent (4) and mix.
11. Dilute to 15 ml with distilled water and mix by inversion.
12. Allow to stand at room temperature away from direct light for 60 minutes.
13. Measure the transmittancy of the sample against the reagent blank set at 100% transmission at a wave length of **700** mμ, obtain the value for uric acid from the calibration table and record.
14. Measure the transmittancy of the 4 mg standard against the reagent blank set at 100% transmission at a wave length of **700** mμ and check against the value in the calibration table.
15. Calculate the concentration of uric acid as follows.

Calculation

If the transmittancy reading obtained in Step 14 is the same as that corresponding to a concentration of 4 mg in the calibration table, no further correction need be made and the value recorded in Step 13 represents the concentration of uric acid in mg per 100 ml sample.

If the transmittancy reading obtained in Step 14 differs from that indicated in the calibration table, the concentration of uric acid is calculated by the following formula:

$$\text{Value from calibration table (from Step 13)} \times \frac{4}{w},$$

where w represents the concentration indicated by the transmittancy reading obtained in Step 14.

CALIBRATION PROCEDURE

Special Apparatus

Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.

Graduated test tubes—10 ml in 0.1 ml; the 5 ml graduation must be accurately calibrated.

Graduated test tubes—25 ml in 0.5 ml; the 15 ml graduation must be accurately calibrated.

Reagents

3. **Cyanide-Urea Reagent (Newton).** Same as for Test Procedure.
4. **Phosphotungstic Acid Reagent (Brown).** Same as for Test Procedure.
5. **Lithium Carbonate Solution.** In a 250 ml Florence flask place 150 ml distilled water and add 0.6 gm lithium carbonate (Li_2CO_3—Baker & Adamson, Reagent); heat to 60° C. shaking frequently; filter at once. This solution is used warm and should be prepared just before needed. Required only for preparation of uric acid stock standard (8a).
6. **Formaldehyde, 40%** (HCHO—Baker & Adamson, Reagent). Required only for preparation of uric acid stock standard (8a).
7. **Sulfuric Acid, Volumetric 1 N.** Slowly add 30 ml concentrated sulfuric acid (H_2SO_4—Grasselli, A.C.S.) to 970 ml distilled water; mix well and allow to cool; standardize against 1 N alkali using phenolphthalein as indicator. Required only for preparation of uric acid stock standard (8a).
8. **Uric Acid Standards**
   (a) Uric acid stock standard, Folin (1 ml contains 1 mg uric acid). In the mouth of a dry 1000 ml volumetric flask insert a dry powder funnel; place 1 gm uric acid ($C_5H_4N_4O_3$—Pfanstiehl, C.P.) in the funnel and tap the funnel so as to transfer practically all of the uric acid to the flask; add 150 ml warm lithium carbonate solution (5) to the flask, rinsing off the weighing crystal and the funnel at the same time; stopper the flask and shake vigorously under running hot tap water until all of the uric acid is dissolved (not more than 5 minutes); immediately shake the flask under running cold tap water until room temperature is reached; add 20 ml 40% formaldehyde (6) and about 500 ml distilled water; add slowly, from a pipette while constantly shaking the flask, 25 ml 1 N sulfuric acid (7); dilute to volume with distilled water and mix thoroughly. Preserve in a brown glass bottle away from direct light. Stability indefinite.
   (b) Dilute standard (1 ml contains 0.025 mg uric acid). In a 100 ml volumetric flask place 2.5 ml stock standard (8a) and dilute to volume with distilled water. Prepare just before use.
   (c) Calibration standards. In a series of six accurately calibrated 10 ml graduated test tubes place respectively 0, 1, 2, 3, 4 and 5 ml of the dilute standard (8b); add 5, 4, 3, 2, 1 and 0 ml distilled water respectively, bringing the volume in each tube to 5 ml. These standards represent concentrations of uric acid equivalent to 0, 2, 4, 6, 8 and 10 mg per 100 ml sample. Prepare just before use and proceed immediately as follows.

Technic

1. Transfer 1 ml of each calibration standard (8c) to a correspondingly marked 25 ml graduated test tube.

2. To each tube add 4 ml distilled water.
3. Proceed exactly as outlined in the Test Procedure, Steps 9–12 inclusive.
4. Set the zero concentration standard at 100% transmission at a wave length of 700 $m\mu$ and record the transmittancy of the 2, 4, 6, 8 and 10 mg per cent standards. The transmittancy of the 10 mg standard should be approximately 19%.
5. Plot the observed values on semi-log graph paper—the points fall on a very slight curve which passes through the origin (Calibration Curve U-15).
6. Prepare a calibration table from the T-C values obtained from the plotted curve.

Method U-15b

DETERMINATION IN WHOLE BLOOD

SAMPLE

Blood withdrawn during post-absorptive state. Anticoagulant—lithium oxalate; avoid an excess. Due to non-uric acid chromogenic substances contained in the red blood cells, analyses are preferably made on plasma or serum. If whole blood is to be used, a special precipitating procedure must be followed.

TEST PROCEDURE

Special Apparatus

As listed under the Test Procedure for plasma or serum.

Reagents

3. **Cyanide-Urea Reagent** (Newton). Same as for plasma or serum.
4. **Phosphotungstic Acid Reagent** (Brown). Same as for plasma or serum.
9. **Tungstomolybdate Solution** (Benedict-Behre). In a 125 ml beaker place 10 gm molybdic acid (MoO_3—Mallinckrodt, A.C.S., Ammonia-free) and slowly add 50 ml 1 N sodium hydroxide (a); boil for 4–5 minutes; filter while hot; wash the residue on the filter with 150 ml hot distilled water; cool filtrate and washing; transfer to a 1000 ml volumetric flask; add 600 ml sodium tungstate solution (b); dilute to volume with distilled water and mix thoroughly. Stability indefinite.
   - (a) Sodium hydroxide, volumetric 1 N; prepare by diluting slightly more than the required amount of concentrated carbonate-free sodium hydroxide solution (see Appendix) and standardize against 1 N acid using phenolphthalein as indicator. Preserve in ceresin-lined bottle.
   - (b) Sodium tungstate solution; dissolve 80 gm sodium tungstate ($Na_2WO_4 \cdot 2H_2O$—Mallinckrodt, Folin Special) in 600 ml distilled water.
10. **Sulfuric Acid, Volumetric 0.62 N.** Dilute 620 ml 1 N sulfuric acid (7) to 1000 ml with distilled water and mix.
11. **Acid Lithium Chloride Solution** (Newton). Dissolve 7.5 gm lithium chloride (LiCl—Baker & Adamson, Reagent) in 50 ml distilled water; add 35 ml concentrated hydrochloric acid (HCl—Baker & Adamson, A.C.S.); dilute to 1000 ml with distilled water and mix. Stability indefinite.
12. **Silver Nitrate Solution** (Newton). Dissolve 29 gm silver nitrate ($AgNO_3$—Baker & Adamson, A.C.S.) in 100 ml distilled water and dilute to 1000 ml. Preserve in a brown glass bottle.
13. **Acid Blank Solution** (Newton). Dilute 7 ml concentrated hydrochloric acid (HCl—Baker & Adamson, A.C.S.) to 1000 ml with distilled water and mix.

Technic

Note: In this procedure, in order to use the calibration curve and table prepared as previously outlined for plasma or serum, a standard must be run simultaneously with the unknown to obtain the proper correction factor (see note under technic for plasma or serum).

For concentrations of uric acid in excess of 10 mg per 100 ml, use 8.5 ml distilled water, 0.5 ml sample, 0.5 ml tungstomolybdate solution (9) and 0.5 ml 0.62 N sulfuric acid (10) in Step 1 and multiply the values obtained from the calibration table for plasma or serum by 2.

1. In a 25 ml Erlenmeyer flask place
   - 7 ml distilled water
   - 1 ml sample from an Ostwald-Folin pipette, thoroughly rinsing out the pipette by drawing in and expelling some of the mixture several times
   - 1 ml tungstomolybdate solution (9)
   - 1 ml 0.62 N sulfuric acid (10), added drop by drop, while constantly agitating the flask.
2. Allow to stand for 5 minutes.
3. Filter through a dry 9 cm Whatman #4 paper into a small dry flask.
4. In a 15 ml centrifuge tube place 5 ml filtrate.
5. Add 1 ml acid lithium chloride solution (11) and mix.
6. Add 1 ml silver nitrate solution (12) and shake well.
7. Centrifuge at 1500 rpm for 5 minutes.
8. Decant the clear centrifugate into an accurately graduated 10 ml test tube, allowing time for complete drainage—touch the lip of the centrifuge tube to the test tube to obtain the last drop; dilute to 10 ml with distilled water and mix—a slight opalescence may be noted; this disappears upon the addition of the cyanide.

9. In a 25 ml graduated test tube (sample) place
   5 ml diluted centrifugate
   5 ml distilled water and mix.
10. In a second 25 ml graduated test tube (reagent blank) place
    2.5 ml acid blank solution (13)
    7.5 ml distilled water and mix.
11. In a third 25 ml graduated test tube (standard) place
    1 ml standard uric acid solution (containing 0.01 mg)
    2.5 ml acid blank solution (13)
    6.5 ml distilled water and mix.
12. To each tube add FROM A BURETTE 3 ml freshly prepared cyanide-urea reagent (3) and mix.
13. Add 1 ml phosphotungstic acid reagent (4) and mix.
14. Dilute to 15 ml with distilled water and mix by inversion.
15. Allow to stand at room temperature away from direct light for 60 minutes.
16. Measure the transmittancy of the sample against the reagent blank set at 100% transmission at a wave length of 700 mμ, obtain the value for uric acid from the calibration table for plasma or serum and record.
17. Measure the transmittancy of the 4 mg standard against the reagent blank set at 100% transmission at a wave length of 700 mμ and check against the value in the calibration table for plasma or serum.
18. Calculate the concentration of uric acid as follows.

Calculation

If the transmittancy reading obtained in Step 17 is the same as that corresponding to a concentration of 4 mg in the calibration table for plasma or serum, no further correction need be made and the value recorded in Step 16 represents the concentration of uric acid in mg per 100 ml blood.

If the transmittancy reading obtained in Step 17 differs from that indicated in the calibration table for plasma or serum, the concentration of uric acid is calculated by the following formula:

$$\text{Value from calibration table (from Step 16)} \times \frac{4}{w},$$

where w represents the concentration indicated by the transmittancy reading obtained in Step 17.

Method U-15c

DETERMINATION IN URINE

SAMPLE

Aliquot of a 24-hour specimen.

TEST PROCEDURE

Special Apparatus

As listed under the Test Procedure for plasma or serum.

Reagents

1. **Sulfuric Acid, Volumetric N/12.** Same as for plasma or serum.
2. **Sodium Tungstate, 10% Aqueous.** Same as for plasma or serum.
3. **Cyanide-Urea Reagent (Newton).** Same as for plasma or serum.
4. **Phosphotungstic Acid Reagent (Brown).** Same as for plasma or serum.

Technic

1. Filter a small portion of thoroughly mixed urine—any precipitated urates must be redissolved by gentle heating before filtering.
2. In a 100 ml volumetric flask place 10 ml filtered urine and dilute to volume with distilled water.
3. In a 30 ml Erlenmeyer flask place
   8 ml N/12 sulfuric acid (1)
   1 ml diluted urine from an Ostwald-Folin pipette, thoroughly rinsing out the pipette by drawing in and expelling some of the mixture several times.
4. Mix the contents of the flask by gentle rotation and add 1 ml 10% sodium tungstate (2), drop by drop, while constantly but gently, rotating the flask.
5. Shake the flask vigorously until the mixture is foam-free.
6. Proceed exactly as outlined in the Test Procedure for plasma or serum, Steps 5–12 inclusive.
7. Measure the transmittancy of the sample against the reagent blank set at 100% transmission at a wave length of 700 mμ, multiply the value for uric acid from the calibration table for plasma or serum by 10 and record.
8. Measure the transmittancy of the 4 mg standard against the reagent blank set at 100% transmission at a wave length of 700 mμ and check against the value in the calibration table for plasma or serum.
9. Calculate the concentration of uric acid as follows.

Calculation

If the transmittancy reading obtained in Step 8 is the same as that corresponding to a concentration of 4 mg in the calibration table for plasma or serum, no further correction need be made and the value recorded in Step 7 represents the concentration of uric acid in mg per 100 ml urine.

If the transmittancy reading obtained in Step 8 differs from that indicated in the calibration table for plasma or serum, the concentration of uric acid is calculated by the following formula:

Recorded value from calibration table (from Step 7) $\times \frac{4}{w}$, where w represents the concentration indicated by the transmittancy reading obtained in Step 8.

$$\frac{\text{Volume of 24-hour specimen}}{100} \times \text{Uric acid in mg per 100 ml} = \text{Mg uric acid excreted in 24 hours}$$

PROCEDURE FOR USE WITH LINEAR-SCALE PHOTOELECTRIC COLORIMETERS (SHEARD-SANFORD TYPE)

TEST PROCEDURE

Exactly as outlined; set the reagent blank at 100 and use a red filter.

CALIBRATION PROCEDURE

Exactly as outlined—use a red filter; plot the observed readings on semi-log graph paper—the points fall on a very slight curve passing through the origin.

PROCEDURE FOR USE WITH LOGARITHMIC-SCALE PHOTOELECTRIC COLORIMETERS (KLETT-SUMMERSON TYPE)

TEST PROCEDURE

Exactly as outlined; set the reagent blank at 0 and use a red filter.

CALIBRATION PROCEDURE

Exactly as outlined—use a red filter; plot the observed readings on linear graph paper—the points fall on a very slight curve passing through the origin.

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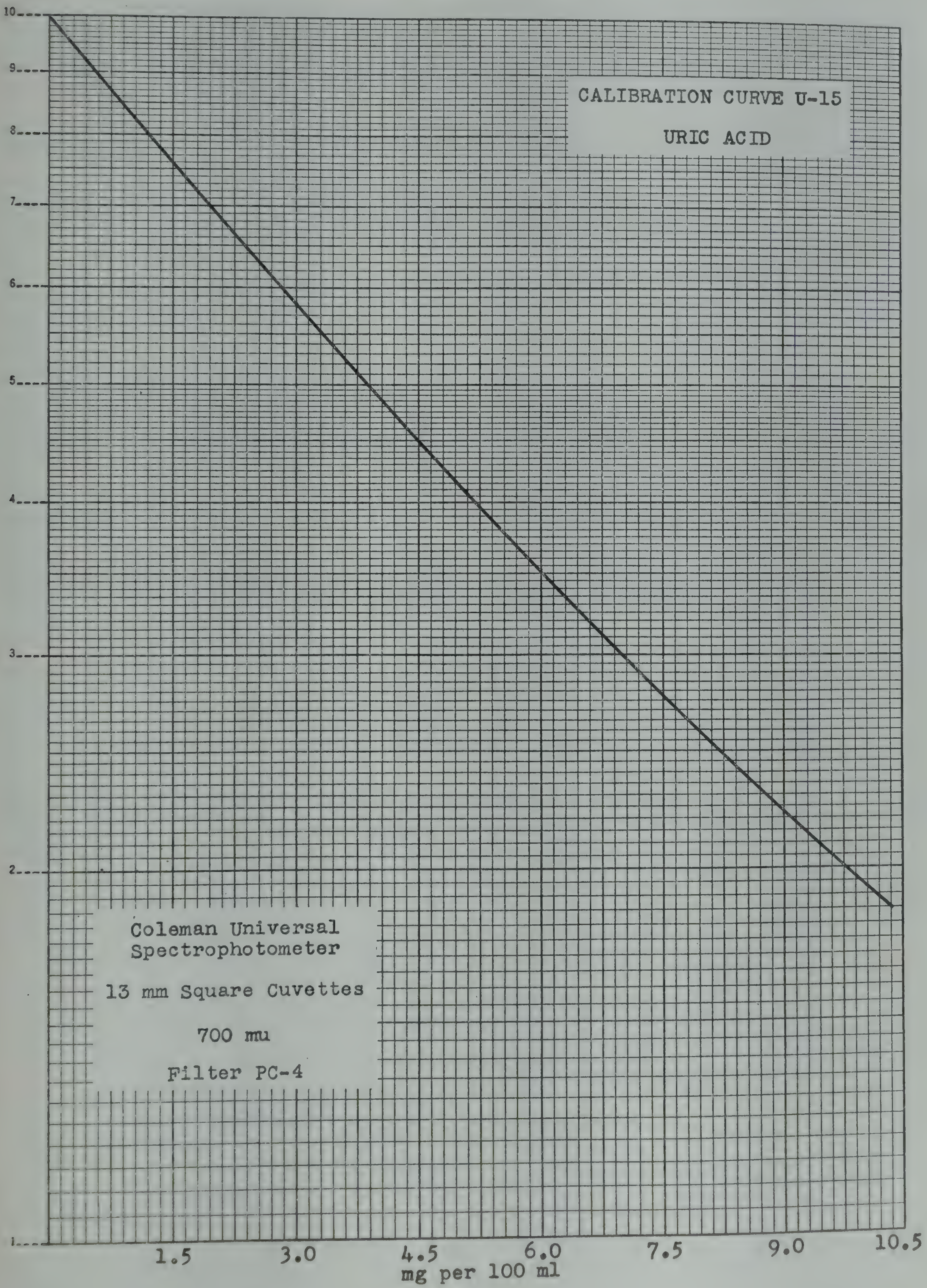
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Coleman Universal Spectrophotometer
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9
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5
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3
2
1
1.5
3.0
4.5
6.0
7.5
9.0
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mg per 100 ml

Meter Reading	0	1	2	3	4	5	6	7	8	9
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50										
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URIC ACID

A spectrophotometric adaptation based on the methods and modifications of Folin and Denis (1912), Folin (1930) and Kern and Stransky (1937)

PRINCIPLE

Uric acid reacts with the tungsten in phosphotungstic acid with a resulting lowering in valence and the formation of a blue complex, the color of which is stabilized by the use of glycerol silicate and polyanethol sodium sulfonate.

DETERMINATION IN PLASMA OR SERUM

SAMPLE

Blood withdrawn during post-absorptive state. Due to the presence of interfering chromogenic substances in the erythrocytes, analyses on plasma or serum are preferable. Anticoagulant—lithium oxalate (potassium oxalate may be used but an excess must be avoided).

TEST PROCEDURE

Special Apparatus

Ostwald-Folin pipettes accurately calibrated "to contain" 1 ml.

Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.

Reagents

1. **Sodium Hydroxide, Volumetric 0.5 N.** Prepare by diluting slightly more than the required amount of concentrated carbonate-free sodium hydroxide solution (see Appendix) and standardize against 1 N acid using phenolphthalein as indicator—10 ml of 1 N acid require 20 ml of 0.5 N alkali. Preserve in ceresin-lined bottle.
2. **Phosphotungstic Acid Reagent (Folin-Denis).** In a 1500 ml Florence flask place 800 ml distilled water; add 100 gm sodium tungstate ($Na_2WO_4 \cdot 2H_2O$—Mallinckrodt, Folin Special) and shake until dissolved; add 80 ml 85% phosphoric acid (H_3PO_4—Baker & Adamson, A.C.S.); mix well; reflux gently for two hours; cool to room temperature; dilute to 1000 ml with distilled water. Filtering, if necessary, should be made through tightly-packed glass wool. Preserve in brown glass-stoppered bottle.
3. **Glycerol Silicate Reagent (Kern-Stransky).** Dissolve 80 gm sodium silicate ($Na_2SiO_3 \cdot 9H_2O$—Baker & Adamson, Reagent) in 800 ml hot distilled water; add 160 ml glycerine ($C_3H_5(OH)_3$—Baker & Adamson, A.C.S.); stir well and allow to cool to room temperature. If a slight trace of turbidity is apparent, filter through a dry Whatman #50 paper. Preserve in ceresin-lined bottle.
4. **Polyanethol Sodium Sulfonate, 2% Aqueous.** Dissolve 1 gm polyanethol sodium sulfonate ("Liquoid"—Hoffmann-LaRoche) in 50 ml distilled water. Preserve in refrigerator.

Technic

1. In a 50 ml Erlenmeyer flask (sample) place
   8 ml distilled water
   1 ml plasma or serum from an Ostwald-Folin pipette, thoroughly rinsing out the pipette by drawing in and expelling some of the mixture several times.
2. In another 50 ml Erlenmeyer flask (reagent blank) place
   9 ml distilled water.
3. To each flask add 0.5 ml 0.5 N sodium hydroxide (1), and mix.
4. Add 0.5 ml phosphotungstic acid reagent (2); adding the first half rapidly and the remainder slowly, drop by drop, while constantly agitating the flask.
5. Allow to stand for 5 minutes.
6. Filter through dry 9 cm Whatman #4 papers into correspondingly marked dry test tubes.
7. In a cuvette place 5 ml sample filtrate.
8. In another cuvette place 5 ml reagent blank filtrate.
9. To each cuvette add 2.5 ml glycerol silicate reagent (3), and mix.
10. Add 0.5 ml 2% polyanethol sodium sulfonate (4), and mix thoroughly.
11. Add 0.5 ml phosphotungstic acid reagent (2), and mix thoroughly.
12. Allow to stand for 15 minutes.
13. Measure the transmittancy of the sample against the reagent blank set at 100% transmission at a wave length of 700 mμ and obtain the concentration of uric acid from the calibration table.

CALIBRATION PROCEDURE

Special Apparatus

Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.

Mixing cylinders—Normax; glass-stoppered, 25 ml in 0.2 ml.

Reagents

1. **Sodium Hydroxide, Volumetric 0.5 N.** Same as for Test Procedure.
2. **Phosphotungstic Acid Reagent (Folin-Denis).** Same as for Test Procedure.
3. **Glycerol Silicate Reagent (Kern-Stransky).** Same as for Test Procedure.

4. **Polyanethol Sodium Sulfonate, 2% Aqueous.** Same as for Test Procedure.
5. **Lithium Carbonate Solution.** In a 250 ml Florence flask place 150 ml distilled water and add 0.6 gm lithium carbonate (Li_2CO_3—Baker & Adamson, Reagent); heat to 60° C. shaking frequently; filter at once. This solution is used warm and should be prepared just before needed. Required only for preparation of uric acid stock standard (8a).
6. **Formaldehyde, 40%.** (HCHO—Baker & Adamson, Reagent). Required only for preparation of uric acid stock standard (8a).
7. **Sulfuric Acid, Volumetric 1 N.** Slowly add 30 ml concentrated sulfuric acid (H_2SO_4—Grasselli, A.C.S.) to 970 ml distilled water; mix well and allow to cool; standardize against 1 N alkali using phenolphthalein as indicator. Required only for preparation of uric acid stock standard (8a).
8. **Uric Acid Standards.**
    (a) Uric acid stock standard, Folin (1 ml contains 1 mg uric acid). In the mouth of a dry 1000 ml volumetric flask insert a dry powder funnel; place 1 gm uric acid ($C_5H_4N_4O_3$—Pfanstiehl, C.P.) in the funnel and tap the funnel so as to transfer practically all of the uric acid to the flask; add 150 ml warm lithium carbonate solution (5) to the flask, rinsing off the weighing crystal and the funnel at the same time; stopper the flask and shake vigorously under running hot tap water until all of the uric acid is dissolved (not more than 5 minutes); immediately shake the flask under running cold tap water until room temperature is reached; add 20 ml 40% formaldehyde (6) and about 500 ml distilled water; add slowly, from a pipette while constantly shaking the flask, 25 ml 1 N sulfuric acid (7); dilute to volume with distilled water and mix thoroughly. Preserve in a brown glass bottle away from direct light. Stability indefinite.
    (b) Dilute standard (1 ml contains 0.05 mg uric acid). In a 100 ml volumetric flask place 5 ml stock standard (8a) and dilute to volume with distilled water. Prepare just before use.
    (c) Calibration standards. In a series of four accurately calibrated 25 ml mixing cylinders place respectively 0, 1, 2 and 4 ml of the dilute standard (8b); add sufficient distilled water to bring the volume in each cylinder to 18 ml and mix. These standards represent concentrations of uric acid equivalent to 0, 2.5, 5 and 10 mg per 100 ml sample. Prepare just before use and proceed immediately as follows.

Technic

1. To each calibration standard (8c) add 1 ml 0.5 N sodium hydroxide (1).
2. Add 1 ml phosphotungstic acid reagent (2).
3. Stopper the cylinders and mix thoroughly.
4. Transfer a 5 ml portion from each cylinder to a correspondingly marked cuvette.
5. Proceed exactly as outlined in the Test Procedure, Steps 9–12 inclusive.
6. Set the zero concentration standard at 100% transmission at a wave length of 700 mμ and record the transmittancy of the 2.5, 5 and 10 mg per cent standards. The transmittancy of the 10 mg standard should be approximately 37.5%.
7. Plot the observed values on semi-log graph paper—the points fall on a straight line which passes through the origin (Calibration Curve U-16).
8. Prepare a calibration table from the T-C values obtained from the plotted curve.

Method U-16b

DETERMINATION IN URINE

SAMPLE

Aliquot of a 24-hour specimen.

TEST PROCEDURE

Special Apparatus

As listed under the Test Procedure for blood.

Reagents

1. **Sodium Hydroxide, Volumetric 0.5 N.** Same as for blood.
2. **Phosphotungstic Acid Reagent (Folin-Denis).** Same as for blood.
3. **Glycerol Silicate Reagent (Kern-Stransky).** Same as for blood.
4. **Polyanethol Sodium Sulfonate, 2% Aqueous.** Same as for blood.

Technic

1. Filter a small portion of thoroughly mixed urine—any precipitated urates must be redissolved by gentle heating before filtering.
2. In a 100 ml volumetric flask place 10 ml filtered urine and dilute to volume with distilled water.
3. In a 50 ml Erlenmeyer flask (sample) place
    8 ml distilled water
    1 ml diluted urine from an Ostwald-Folin pipette, thoroughly rinsing out the pipette by drawing in and expelling some of the mixture several times.

4. Proceed exactly as outlined in the Test Procedure for blood, Steps 2–12 inclusive.
5. Measure the transmittancy of the sample against the reagent blank set at 100% transmission at a wave length of **700** mμ and multiply the value from the calibration table for blood by 10 to obtain the concentration of uric acid in mg per 100 ml urine.

Calculation

$$\frac{\text{Volume of 24-hour specimen}}{100} \times \text{Uric acid in mg per 100 ml} = \text{Mg uric acid excreted in 24 hours}$$

◆

PROCEDURE FOR USE WITH LINEAR-SCALE PHOTOELECTRIC COLORIMETERS (SHEARD-SANFORD TYPE)

TEST PROCEDURE

Exactly as outlined; set the reagent blank at 100 and use a red filter.

CALIBRATION PROCEDURE

Exactly as outlined—use a red filter; plot the observed readings on semi-log graph paper—the points fall on a straight line passing through the origin.

◆

PROCEDURE FOR USE WITH LOGARITHMIC-SCALE PHOTOELECTRIC COLORIMETERS (KLETT-SUMMERSON TYPE)

TEST PROCEDURE

Exactly as outlined; set the reagent blank at 0 and use a red filter.

CALIBRATION PROCEDURE

Exactly as outlined—use a red filter; plot the observed readings on linear graph paper—the points fall on a straight line passing through the origin.

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Kern, A., and Stransky, E., "Beitrag zur kolorimetrischen Bestimmung der Harnsäure," *Biochem. Zeit.*, 290, 419 (1937).

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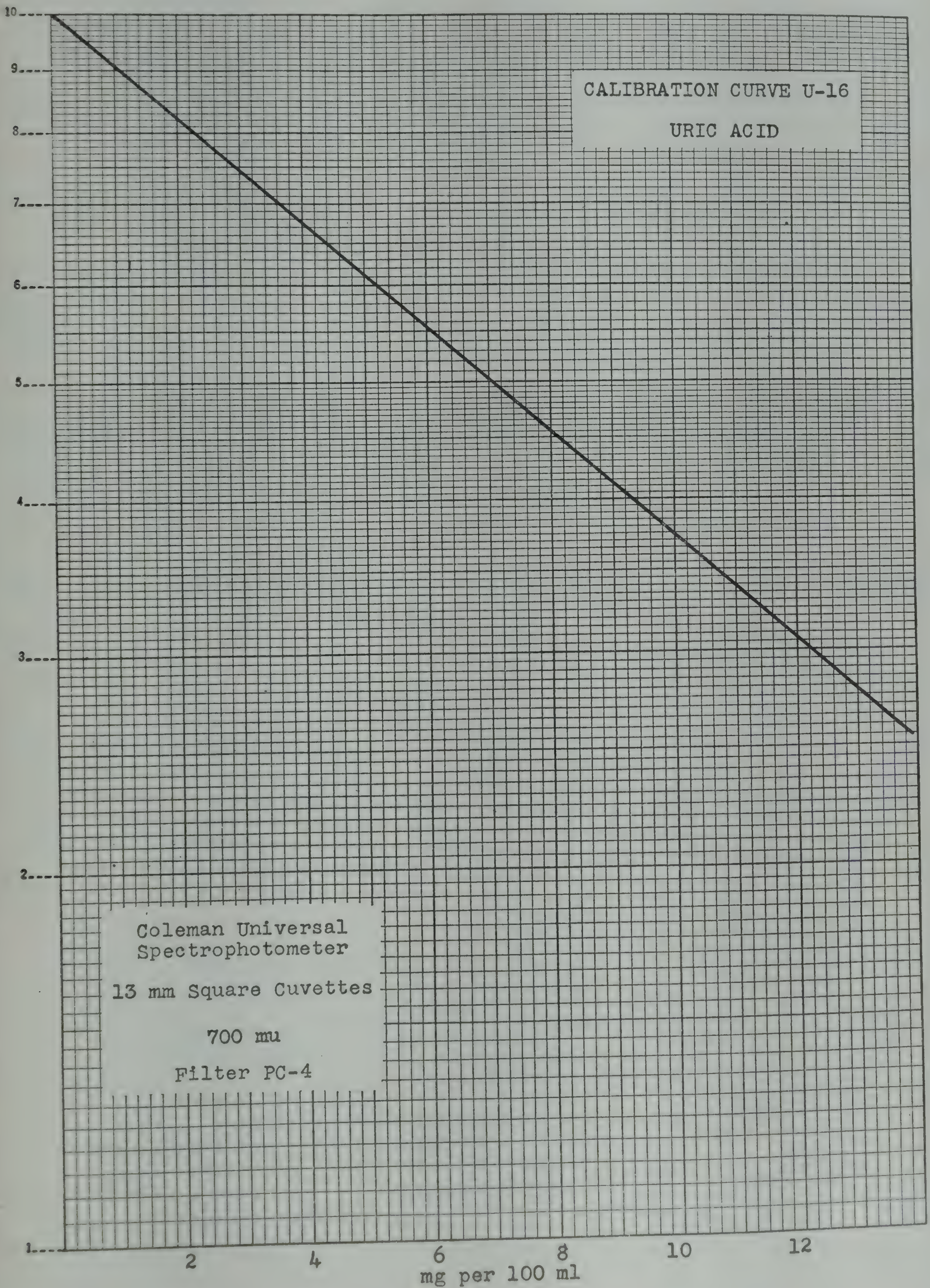
NOTES

NOTES


CALIBRATION CURVE U-16
URIC ACID
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9
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Coleman Universal Spectrophotometer
13 mm Square Cuvettes
700 mu
Filter PC-4
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UROBILINOGEN

A spectrophotometric adaptation based on the methods and modifications of Terwen (1925), Sparkman (1936), Watson (1936; 1937), Naumann (1936), Schwartz, Sborov and Watson (1944), Watson, Schwartz, Sborov and Bertie (1944), Watson and Hawkinson (1947), Voegtlin (1948) and McDonald and Kelley (1948)

PRINCIPLE

The colorless chromogen of urobilin, urobilinogen, upon treatment with p-dimethylaminobenzaldehyde in hydrochloric acid solution forms urobilinogen-aldehyde, a condensation product with a reddish violet color (Ehrlich reaction), the intensity of which is quantitatively proportional to the amount of Ehrlich-reacting substances present in the sample. Sodium acetate is used to convert the excess hydrochloric acid of the Ehrlich reagent into acetic acid and to destroy the color produced by indol. Other nonurobilinogen substances also produce a very slight color but this is insignificant from a clinical standpoint. With the simplified technics (Methods U-20a and U-20c) results are expressed in terms of Ehrlich Units since it is assumed that the majority of the color is due to urobilinogen, whereas, in the exact quantitative procedures (Methods U-20b and U-20d) results are expressed in mg per 100 ml (or 100 gm) sample. One Ehrlich Unit is defined as the amount of color that is produced by 1 mg of crystalline urobilinogen, assuming that all the color is due to urobilinogen.

DETERMINATION IN URINE

SAMPLE

Aliquot of a 24-hour specimen collected in a brown glass bottle containing 5 gm sodium carbonate and 40 ml petroleum ether and kept in a cool dark place. Acetone and nitrites must not be present. In samples containing an excess of bilirubin or protein and where the most accurate results are required, the ferrous hydroxide-petroleum ether extraction method (Method U-20b) must be employed.

TEST PROCEDURE

A. In the Absence of Bilirubin and/or Protein

Special Apparatus

Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.

Reagents

1. **Ehrlich Aldehyde Reagent (Watson).** In a 500 ml mixing cylinder place 150 ml concentrated hydrochloric acid (HCl—Baker & Adamson, A.C.S.); add 100 ml distilled water and mix; add 0.7 gm p-dimethylaminobenzaldehyde ($C_9H_{11}OH$—Reagent Chemicals, Special) and shake until dissolved. The reagent should show no more than a faint yellow color. Preserve in brown glass-stoppered bottle.
2. **Sodium Acetate, Saturated Aqueous.** In an 8 ounce bottle place 130 gm finely pulverized crystalline sodium acetate ($NaC_2H_3O_2 \cdot 3H_2O$—Baker & Adamson, A.C.S.) and add 100 ml distilled water previously heated to 45° C.; shake thoroughly and allow to cool and settle. Use the clear supernatant fluid. Note: Complete saturation is essential.

Technic

Note: It is absolutely essential that in Steps 2 and 3, the reagents be added in the order named. The determination should be carried out with adequate protection against bright direct, reflected or diffused daylight.

For concentrations of urobilinogen in excess of 2.4 units per 100 ml, dilute the filtered urine with an equal volume of distilled water before proceeding with Step 2 and multiply the values obtained from the calibration table by 8 when calculating the concentration.

1. Filter a small ether-free portion of thoroughly mixed urine.
2. In a cuvette (sample) place
   - 2.5 ml filtered urine
   - 2.5 ml aldehyde reagent (1), mix thoroughly and IMMEDIATELY add
   - 5.0 ml saturated sodium acetate (2) and mix thoroughly.
3. In another cuvette (sample blank) place
   - 2.5 ml filtered urine
   - 5.0 ml saturated sodium acetate (2) and mix
   - 2.5 ml aldehyde reagent (1), added slowly while constantly agitating the cuvette.
4. Within 5 minutes, measure the transmittancy of the sample against the sample blank set at 100% transmission at a wave length of 565 mμ, obtain the value from the calibration table and calculate the urobilinogen in terms of Ehrlich Units per 100 ml sample.

Calculation

Value obtained from calibration table × 4 = Concentration of urobilinogen in Ehrlich Units per 100 ml

$$\frac{\text{Urobilinogen in E.U. per 100 ml} \times \text{Volume of 24-hour specimen}}{100} = \text{Urobilinogen in E.U. excreted in 24 hours}$$

Method U-20b

B. In the Presence of Bilirubin and/or Protein

Special Apparatus

Separatory funnels, pear-shaped, 125 ml and 250 ml. Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.

Reagents

1. **Erlich Aldehyde Reagent (Watson).** Same as for Test Procedure A.
2. **Sodium Acetate, Saturated Aqueous.** Same as for Test Procedure A.
3. **Ferrous Sulfate, 20% Aqueous.** Dissolve 20 gm pulverized crystalline ferrous sulfate ($FeSO_4 \cdot 7H_2O$—Baker & Adamson, Reagent) in 92 ml distilled water. Prepare just before use.
4. **Sodium Hydroxide, Volumetric 2.5 N.** Prepare by diluting slightly more than the required amount of concentrated carbonate-free sodium hydroxide solution (see Appendix); dilute 20 ml of this approximately 2.5 N solution to 100 ml; titrate against standard 0.5 N acid using phenolphthalein as indicator; adjust accordingly and re-titrate—20 ml of 0.5 N acid require 20 ml of a 1:5 dilution of 2.5 N alkali. Preserve in ceresin-lined bottle.
5. **Petroleum Ether, Special Purified (B.P. 30–60° C.).** Place 500 ml petroleum ether (Baker & Adamson, A.C.S.) in a beaker, place in a desiccator and allow to stand over sulfuric acid for several days; wash repeatedly with distilled water and redistil. Preserve in a brown glass bottle with tight-fitting aluminum-lined plastic screw cap.
6. **Acetic Acid, Glacial** (CH_3COOH—Baker & Adamson, A.C.S.).

Technic

Note: Steps 4–18 inclusive should be carried out in the absence of bright light.

1. In a 250 ml Erlenmeyer flask place 50 ml thoroughly mixed but unfiltered urine.
2. Add 25 ml freshly prepared 20% ferrous sulfate (3), mix, add 25 ml 2.5 N sodium hydroxide (4) and mix thoroughly.
3. Stopper the flask and allow to stand in a warm dark place for 1–2 hours.
4. Mix thoroughly and filter.
5. Perform a qualitative test on the filtrate as follows:

   Mix 2 ml filtrate with an equal volume aldehyde reagent (1) and immediately add 4 ml saturated sodium acetate (2)—if the resultant color is an intense red, use 2 ml filtrate in Step 6; if pale red, use 10 ml; if faint pink, use 25 ml; if no color appears, use 50 ml.
6. Place the indicated amount of filtrate (as determined in Step 5) in a 125 ml pear-shaped separatory funnel—if less than 25 ml filtrate is used, make up to this volume with distilled water.
7. Add 50 ml petroleum ether (5) and 5 ml glacial acetic acid (6).
8. Shake vigorously for 1 minute—allow to stand until the layers separate (emulsions may be broken by the further addition of acetic acid or 1–2 ml 95% ethyl alcohol).
9. Decant the petroleum ether extract (through the top of the funnel) into a 250 ml pear-shaped separatory funnel.
10. Extract the aqueous phase in the first funnel twice more with 25 ml portions of petroleum ether (5), in each case decanting the ether extract into the 250 ml funnel.
11. Add 25 ml distilled water to the combined ether extracts and shake for 1 minute.
12. Allow to stand until the layers separate, draw off the lower aqueous phase and discard.
13. Add 2 ml aldehyde reagent (1) and shake vigorously for 1 minute.
14. Add 6 ml saturated sodium acetate (2) and shake vigorously for 1 minute.
15. Allow to stand until the layers separate and quantitatively draw off the lower colored aqueous phase into a 100 ml mixing cylinder (sample).
16. Repeat Steps 13–15 inclusive, until the aqueous layer is colorless or shows no more than a faint tinge of color, combining the aqueous phases in the 100 ml cylinder—usually no more than three extractions are necessary.
17. Dilute the combined urobilinogen-aldehyde solution in the mixing cylinder with distilled water to a volume that will permit the transmittancy reading of the resultant colored solution to fall within the limits of the calibration curve.
18. In another 100 ml mixing cylinder (reagent blank) place the same volume of aldehyde reagent (1) and saturated sodium acetate (2) that were required in Steps 13–16 inclusive and dilute with distilled water to the same volume as in Step 17.
19. Measure the transmittancy of the sample against the reagent blank set at 100% transmission at a wave length of **565 mμ**, obtain the value from the calibration table and calculate the concentration of urobilinogen in mg per 100 ml sample.

Calculation

$$\text{Value obtained from calibration table} \times 2 \times \frac{\text{Volume of final colored solution (in Step 17)}}{\text{Volume of filtrate taken (in Step 6)}} = \text{Concentration of urobilinogen in mg per 100 ml urine}$$

$$\frac{\text{Urobilinogen in mg per 100 ml} \times \text{Volume of 24-hour specimen}}{100} = \text{Mg urobilinogen excreted in 24 hours}$$

CALIBRATION PROCEDURE

Special Apparatus

Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.

Reagents

7. **Acetic Acid, 0.5% Aqueous.** Dilute 5 ml glacial acetic acid (6) to 1000 ml with distilled water.
8. **Urobilinogen Standards**
   - (a) Urobilinogen stock color standard (100 mg per cent dye content). In a 1000 ml volumetric flask place 95 mg Pontacyl violet 6R 150% (DuPont) and 5 mg Pontacyl Carmine 2B (DuPont); add approximately 900 ml 0.5% acetic acid (7) and shake until dissolved; dilute to volume with same. Preserve with a few drops of chloroform.
   - (b) Calibration standards. In a series of eleven accurately calibrated 100 ml volumetric flasks place respectively 0, 0.85, 1.7, 3.4, 5.1, 6.8, 8.5, 10.2, 13.6, 17.0 and 20.4 ml of the stock standard (8a) and dilute to volume with 0.5% acetic acid (7). These standards are respectively equivalent in color to 0, 0.25, 0.05, 0.10, 0.15, 0.20, 0.25, 0.3, 0.4, 0.5 and 0.6 mg urobilinogen per 100 ml sample. Prepare just before use.

Technic

1. Transfer suitable portions of each calibration standard (8b) to correspondingly marked cuvettes.
2. Set the zero concentration standard at 100% transmission at a wave length of 565 mμ and record the transmittancy of the 0.025, 0.05, 0.10, 0.15, 0.20, 0.25, 0.3, 0.4, 0.5 and 0.6 mg per cent standards. The transmittancy of the 0.6 mg standard should be approximately 24%.
3. Plot the observed values on semi-log graph paper—the points fall on a very slight curve which passes through the origin (Calibration Curve U-20).
4. Prepare a calibration table from the T-C values obtained from the plotted curve.

Method U-20c

DETERMINATION IN FECES

SAMPLE

Individual fecal specimens may be used; it is preferable, however, to use an aliquot of a mixed 4-day collection (essential for Method U-20d). The sample is collected and kept in a cool place in a covered paper container (such as a quart-size waxed paper carton) of known weight. After collection is completed, the container and specimen is weighed and the weight of the feces is obtained by difference. For exacting work the petroleum ether extraction method (Method U-20d) must be employed.

TEST PROCEDURE

C. Simplified Procedure

Special Apparatus

Waring blender—if this apparatus is not available, the more cumbersome alternative procedure described in the note may be used.

Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.

Reagents

1. **Ehrlich Aldehyde Reagent (Watson).** Same as for urine.
2. **Sodium Acetate, Saturated Aqueous.** Same as for urine.
3. **Ferrous Sulfate, 20% Aqueous.** Same as for urine.
4. **Sodium Hydroxide, Volumetric 2.5 N.** Same as for urine.

Technic

Note: The entire procedure should be carried out with minimum exposure to bright direct, reflected or diffused daylight. If the feces is obviously acholic, use only 50 ml water in Step 6 and only 25 ml each of ferrous sulfate and sodium hydroxide in Steps 5 and 7 respectively and multiply the value from the calibration table for urine by 100 when calculating the concentration.

For concentrations of urobilinogen in excess of 120 units per 100 gm, use 2 gm feces in Step 2 and multiply the values from the calibration table for urine by 1000 when calculating the concentration.

1. Thoroughly mix the feces in the collection carton.
2. Weigh out 10 gm on a 7 cm filter paper.
3. Place the paper in the container of a Waring blender and add 150 ml distilled water.
4. Mix in the blender for 5 minutes.
5. Decant into a 1000 ml Erlenmeyer flask containing 100 ml 20% ferrous sulfate (3).
6. Rinse out the blender container with another 150 ml portion of distilled water, decanting the washing into the Erlenmeyer flask.
7. While constantly and gently agitating the flask, slowly add 100 ml 2.5 N sodium hydroxide (4).
8. Stopper the flask, mix thoroughly and allow to stand in a warm dark place for 1–2 hours.

Note: If the supernatant fluid is not practically colorless continue the reduction for another 1–2 hours; if reduction is still not complete (as may occur with excessive quantities of urobilinogen), proceed with Step 9 and mix a measured portion of the filtrate with fresh ferrous hydroxide as outlined above and after another reduction period of 1–2 hours, proceed with Step 9.

9. Mix thoroughly and filter (occasionally centrifugation may be necessary to secure a clear fluid).
10. In a cuvette (sample) place
    2.5 ml filtrate
    2.5 ml aldehyde reagent (1), mix thoroughly and IMMEDIATELY add
    5.0 ml saturated sodium acetate (2) and mix thoroughly.
11. In another cuvette (sample blank) place
    2.5 ml filtrate
    5.0 ml saturated sodium acetate (2) and mix
    2.5 ml aldehyde reagent (1), added slowly while constantly agitating the cuvette.
12. Within 5 minutes, measure the transmittancy of the sample against the sample blank set at 100% transmission at a wave length of 565 mμ, obtain the value from the calibration table for urine and calculate the urobilinogen in terms of Ehrlich Units per 100 gm sample.

Calculation

Value obtained from calibration table × 200 = Concentration of urobilinogen in Ehrlich Units per 100 gm feces

$$\frac{\text{Urobilinogen in E.U. per 100 gm} \times \text{Weight of total sample}}{100 \times \text{Number of days}} = \text{Urobilinogen in E.U. excreted in 24 hours}$$

Note: If a Waring blender is not available, extraction may be carried out as follows: Thoroughly mix the feces in the collection carton; weigh out 10 gm on a small piece of waxed paper—if the feces are not sufficiently dry, weigh in a small evaporating dish; transfer the feces to a mortar with 10 ml distilled water and thoroughly grind into a paste; add 80 ml distilled water and grind again until a uniform mixture is obtained; allow to stand until the coarser particles have settled; decant the supernatant fine suspension into a 1000 ml Erlenmeyer flask containing 100 ml ferrous sulfate; the remaining material in the mortar is again ground with small amounts of distilled water, all of which are poured into the Erlenmeyer flask until a total of 200 ml more has been used, the mortar finally being washed clean. If the specimen is obviously acholic, the total amount of water used should not exceed 100 ml and due correction made when calculating. Proceed as outlined in the foregoing procedure, Steps 7–12 inclusive or as outlined in the following procedure, Steps 7–24 inclusive.

Method U-20d

D. Ether Extraction Procedure

Special Apparatus

Waring blender—if this apparatus is not available, the more cumbersome alternative procedure described in the note may be used.

Separatory funnels, pear-shaped, 125 ml and 250 ml.

Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.

Reagents

1. **Ehrlich Aldehyde Reagent (Watson).** Same as for urine.
2. **Sodium Acetate, Saturated Aqueous.** Same as for urine.
3. **Ferrous Sulfate, 20% Aqueous.** Same as for urine.
4. **Sodium Hydroxide, Volumetric 2.5 N.** Same as for urine.
5. **Petroleum Ether, Special Purified.** Same as for urine.
6. **Acetic Acid, Glacial.** Same as for urine.

Technic

Note: The entire procedure should be carried out with minimum exposure to bright direct, reflected on diffused daylight.

1. Thoroughly mix the feces in the collection carton.
2. Weigh out 10 gm on a 7 cm filter paper.
3. Place the paper in the container of a Waring blender and add 150 ml distilled water.
4. Mix in the blender for 5 minutes.
5. Decant into a 1000 ml Erlenmeyer flask containing 100 ml 20% ferrous sulfate (3).

6. Rinse out the blender container with another 150 ml portion of distilled water, decanting the washing into the Erlenmeyer flask.
7. While constantly and gently agitating the flask, slowly add 100 ml 2.5 N sodium hydroxide (4).
8. Stopper the flask, mix thoroughly and allow to stand in a warm dark place for 1–2 hours.

   Note: See note under Step 8, Method U-20c.

9. Mix thoroughly and filter (occasionally centrifugation may be necessary to secure a clear fluid).
10. Perform a qualitative test on the filtrate as follows:

    Mix 2 ml filtrate with an equal volume aldehyde reagent (1) and immediately add 4 ml saturated sodium acetate (2)—if the resultant color is an intense red, use 2 ml filtrate in Step 11; if pale red, use 10 ml; if faint pink, use 25 ml; if no color appears, use 50 ml.

11. Place the indicated amount of filtrate (as determined in Step 10) in a 125 ml pear-shaped separatory funnel—if less than 25 ml filtrate is used, make up to this volume with distilled water.
12. Add 50 ml petroleum ether (5) and 5 ml glacial acetic acid (6).
13. Shake vigorously for 1 minute—allow to stand until the layers separate (emulsions may be broken by the further addition of acetic acid or 1–2 ml 95% ethyl alcohol).
14. Decant the petroleum ether extract (through the top of the funnel) into a 250 ml pear-shaped separatory funnel.
15. Extract the aqueous phase in the first funnel twice more with 25 ml portions of petroleum ether (5), in each case decanting the ether extract into the 250 ml funnel.
16. Add 25 ml distilled water to the combined ether extracts and shake for 1 minute.
17. Allow to stand until the layers separate, draw off the lower aqueous phase and discard.
18. Add 2 ml aldehyde reagent (1) and shake vigorously for 1 minute.
19. Add 6 ml saturated sodium acetate (2) and shake vigorously for 1 minute.
20. Allow to stand until the layers separate and quantitatively draw off the lower colored aqueous phase into a 100 ml mixing cylinder (sample).
21. Repeat Steps 18–20 inclusive, until the aqueous layer is colorless or shows no more than a faint tinge of color, combining the aqueous phases in the 100 ml cylinder—usually no more than three extractions are necessary.
22. Dilute the combined urobilinogen-aldehyde solution in the mixing cylinder with distilled water to a volume that will permit the transmittancy reading of the resultant colored solution to fall within the limits of the calibration curve.
23. In another 100 ml mixing cylinder (reagent blank) place the same volume of aldehyde reagent (1) and saturated sodium acetate (2) that were required in Steps 18–21 inclusive and dilute with distilled water to the same volume as in Step 22.
24. Measure the transmittancy of the sample against the reagent blank set at 100% transmission at a wave length of 565 mμ, obtain the value from the calibration table for urine and calculate the concentration of urobilinogen in mg per 100 gm sample.

Calculation

$$\text{Value obtained from calibration table} \times 50 \times \frac{\text{Volume of final colored solution (in Step 22)}}{\text{Volume of filtrate taken (in Step 11)}} = \text{Concentration of urobilinogen in mg per 100 gm feces}$$

$$\frac{\text{Urobilinogen in mg per 100 gm} \times \text{Weight of total sample}}{100 \times \text{Number of days}} = \text{Mg urobilinogen excreted in 24 hours}$$

PROCEDURE FOR USE WITH LINEAR-SCALE PHOTOELECTRIC COLORIMETERS (SHEARD-SANFORD TYPE)

TEST PROCEDURE

Exactly as outlined; set the sample or reagent blank (according to the method followed) at 100 and use a green filter.

CALIBRATION PROCEDURE

Exactly as outlined—use a green filter; plot the observed readings on semi-log graph paper—the points fall on a very slight curve passing through the origin.

PROCEDURE FOR USE WITH LOGARITHMIC-SCALE PHOTOELECTRIC COLORIMETERS (KLETT-SUMMERSON TYPE)

TEST PROCEDURE

Exactly as outlined; set the sample or reagent blank (according to the method followed) at 0 and use a green filter.

CALIBRATION PROCEDURE

Exactly as outlined—use a green filter; plot the observed readings on linear graph paper—the points fall on a slight curve passing through the origin.

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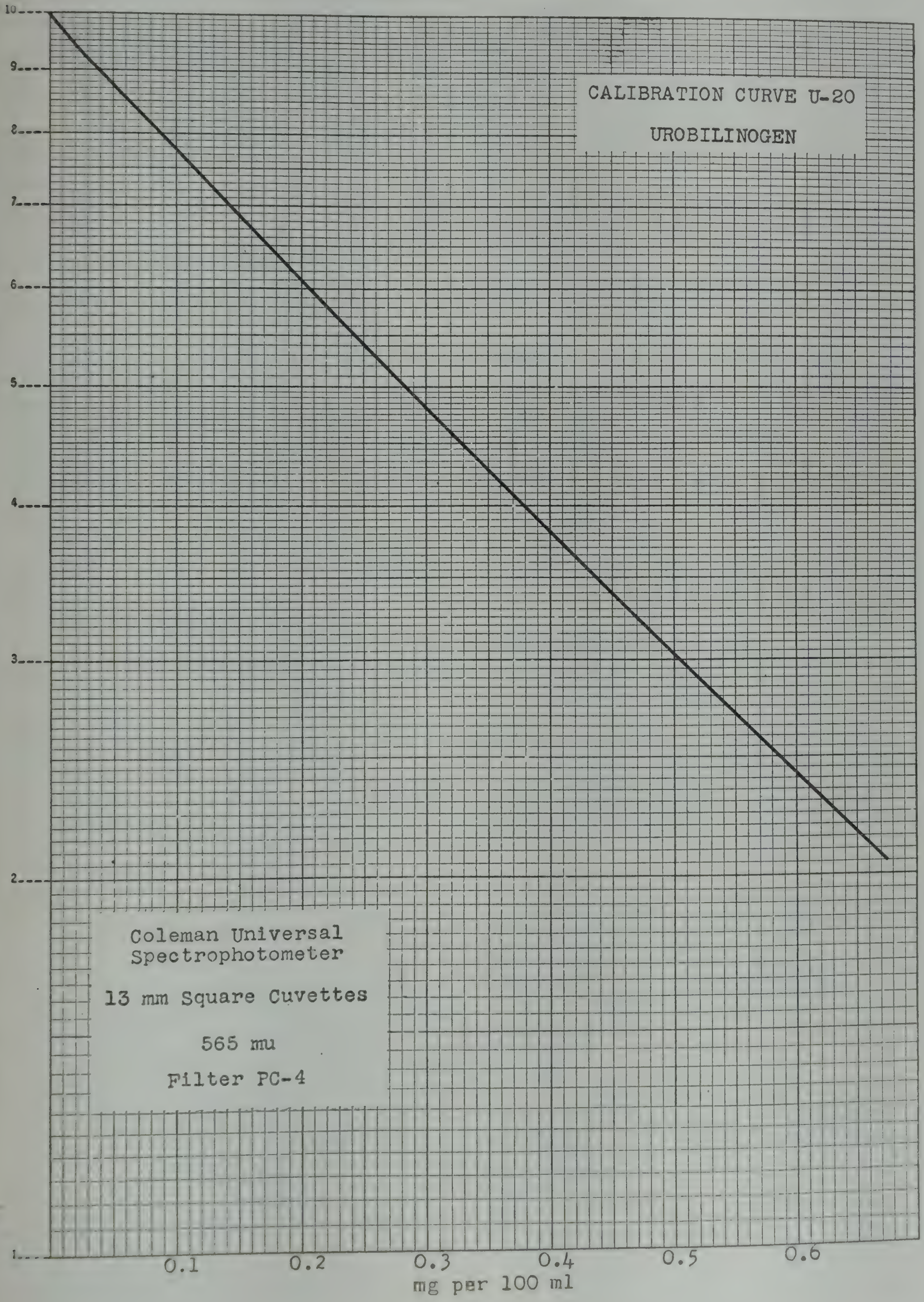
NOTES


CALIBRATION CURVE U-20
UROBILINOGEN
Coleman Universal Spectrophotometer
13 mm Square Cuvettes
565 mu
Filter PC-4
10
9
8
7
6
5
4
3
2
1
0.1
0.2
0.3
0.4
0.5
0.6
mg per 100 ml

Meter Reading	0	1	2	3	4	5	6	7	8	9
0										
10										
20										
30										
40										
50										
60										
70										
80										
90										

BLOOD VOLUME

A spectrophotometric adaptation based on the methods and modifications of Gregersen, Gibson and Stead (1935), Gibson and Evans (1937), Gregersen (1938) and Gibson and Evelyn (1938) as published in "Clinical Methods for the Coleman Junior Clinical Spectrophotometer" and included in this manual by the courtesy of Coleman Instruments, Inc.

PRINCIPLE

Blood volume is determined by measuring the change of plasma optical density resulting from intravenous injection of 15 mg of Evans blue (T-1824) in normal saline. In blood plasma this dye exhibits maximum absorption at **610** mμ and at this wave length follows the Lambert-Beer law to concentration ranges well beyond any clinical requirement of the method.

Dye T-1824, like vital red, appears to combine with the plasma proteins, since the injected portions initially go only to the blood plasma and escape from the blood stream very slowly. This dye also shows slightly different absorption characteristics in water and in plasma solutions and as a consequence the method must originally be calibrated with solutions consisting of known proportions of T-1824 in plasma. Since plasma alone exhibits appreciable absorption at the specified wave length, **610** mμ, the reference must always consist of the same plasma as the sample, less the dye.

Using the Coleman Model 6A Spectrophotometer and 6-310 cuvettes, minima of 1 ml each of reference and sample plasma are required and while clear plasma is preferable a slight lipemia usually will not introduce serious error. The accuracy of the method is not affected if the reference plasma contains up to 15 mg per liter of dye from previous determinations.

The method is capable of high precision provided:

A. The syringe is accurately calibrated and the exact volume is injected.
B. All of the injected solution enters the blood stream.
C. Lipemia is absent.
D. Hemolysis is avoided.

Reagents

1. **Standard Solution of T-1824—Ampules.** Solutions containing identical concentration by weight of different samples of T-1824 dye have been found to differ significantly in optical density (D). In the present method error, both from this source and from variable cuvette dimensions, is eliminated by basing the calibration table (1) on an intravenous solution exhibiting "standard" D when measured in a "standard" 6-310 cuvette. When making a blood volume determination the "actual" D of a specified dilution of the injecting solution is then measured in the same cuvette that will hold the dyed plasma sample. If the "actual" D and "standard" D values differ, the error is eliminated by multiplying the "apparent" plasma volume by the corresponding correction factor "K" appearing in Table V-50B.

   The present calibration is based on a "standard" solution whose 1-1000 dilution in distilled water has an optical density of exactly 0.30 when measured with a Coleman Model 6A Junior Spectrophotometer at **610** mμ, using a standard 6-310 cuvette and with water as reference. This "standard" solution is available in 5 ml ampules and since a maximum of 3 ml is required for injection there is ample remainder for verification in those instances where extreme accuracy demands application of the "K" correction factor.

2. **Standard Solution of T-1824—Preparation.** Dye T-1824 is also available in powder form as EK #3873. Prepare the "standard" solution by transferring 525 mg of the thoroughly dried dye and 900 mg of sodium chloride (U.S.P.) to a 100 ml volumetric flask. Dissolve and then fill to the line with pyrogen free triple distilled water. Mix thoroughly, remove 1 ml and dilute exactly 1-1000 with distilled water. Measure the optical density D of this dilution at **610** mμ with water as reference and preferably using the same cuvette that will retain plasma sample during subsequent blood volume determinations. Correct the concentration of the remaining 99 ml of concentrated solution by adding S ml of pyrogen free normal saline solution (0.9% NaCl).

$$S = \frac{99D}{0.30} - 99 \qquad (1)$$

   Mix thoroughly, filter, sterilize and verify the new concentration by a second measurement of a 1-1000 dilution.

3. **Anticoagulant.** Add 12 gm of ammonium oxalate and 8 gm of potassium oxalate to a 1 liter flask. Dissolve and bring to the mark with distilled water.

Method

Note: To minimize the effects of lipemia this determination is best made after a 12 hours fast, although this precaution is not imperative. It is imperative that every spectrophotometric measurement of a dyed plasma be made against a reference plasma taken just prior to the injection of the dye, as explained below.

Scrupulously avoid hemolysis. Blood samples must be taken only with dry needles and syringes and transferred promptly to tubes containing a dry anticoagulant. A concentration of 2 mg of heparin per ml of blood is recommended or the tube may be prepared for the 10 ml blood sample by evaporating to dryness therein 1 ml of the specified oxalate solution (3).

A. Take 10 ml of blood from the right arm and transfer to an oxalated or heparinized tube, rotating at once to insure immediate solution of the anticoagulant. The plasma from this becomes the reference.

B. Fill a 5 ml calibrated syringe nearly full of the "standard" dye solution, reject any bubbles and bring the plunger exactly to the 3 ml mark, returning the rejected portion of the dye to the ampule if its concentration is to be verified.

C. Find a vein in the right arm and inject exactly 3 ml (15 mg dye) of the "standard" dye solution, making certain that all of the dye enters the vein and taking at least 1 minute for the injection.

Note: If patient weighs less than 50 pounds inject exactly 1 ml of "standard" dye solution and multiply by 0.333 the indicated plasma volume V. If patient weighs between 50 and 100 pounds inject exactly 2 ml of the "standard" dye solution and multiply by 0.667 the indicated plasma volume V.

D. Wait 10 minutes.

E. Take 10 ml of blood from the left arm and transfer to a second oxalated or heparinized tube. The plasma from this becomes the sample.

F. Centrifuge the two blood portions thoroughly and then transfer the resulting plasmas to 6-310 cuvettes, rejecting however if either shows hemolysis or pronounced lipemia.

Note: A 1-200 dilution of whole normal blood in distilled water shows D = 0.065 at 610 mμ after hemolysis is complete. With the recommended method D = 0.198 at a plasma volume of 4 liters, with hemolysis absent. Hence the hemolysis of $^1/_2$% of the RBC in either sample or reference blood could cause an error of roughly $^{65}/_{198}$ or 32%. It is because of this fact that hemolysis must be absolutely avoided.

G. Measure the D of the sample cuvette against the reference cuvette at **610** mμ.

H. From Table V-50A read the "apparent" plasma volume "V" corresponding to the measured D value. (Note that V is determined by the difference in dye concentration between reference an sample.)

I. Using a precision pipette transfer exactly 1.0 ml of the same "standard" dye solution to a 1000 ml volumetric flask and fill to the mark with distilled water. Mix thoroughly, transfer a portion to the identical 6-310 cuvette used in examining the plasma sample and measure Ds at **610** mμ using water as reference. Find in Table V-50B the correction factor "K" corresponding to Ds. Multiply the "apparent" plasma volume by this factor to obtain the actual plasma volume, "P."

$$P = KV \tag{2}$$

Note: Step I and the "K" correction will normally be required only when maximum precision is necessary. In any event, if the "K" factor for a specific cuvette and "standard" dye solution is known from previous tests, equation (2) may be safely applied with only occasional verification of "K."

J. Compute blood volume "B" as follows:

$$B = \frac{P}{(1 - H)} \tag{3}$$

(where B = blood volume in liters;
H = hematocrit of sample)

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NOTES

TABLE V-50A

Optical Density "D" vs. Apparent Plasma Volume "V"

$$V = \frac{0.790^*}{D} \qquad (4)$$

Note: This table is based on the injection of exactly 3.0 ml of a "Standard solution" of T-1824 in 0.9% NaCl whose 1-1000 dilution with distilled water shows D = 0.30 at **610** mμ with the particular 6-310 cuvette used to hold the plasma sample. If the D of the dye-cuvette combination differs appreciably from 0.30 determine the "actual" plasma volume, P, by multiplying the "apparent" plasma volume V, appearing in this table by the corresponding correction factor K of Table V-50B. Details appear under "method."

Optical Density D	Apparent Plasma Volume V	Optical Density D	Apparent Plasma Volume V	Optical Density D	Apparent Plasma Volume V
0.08	9.87	0.32	2.47	0.56	1.41
0.09	8.78	0.33	2.39	0.57	1.39
0.10	7.90	0.34	2.32	0.58	1.36
0.11	7.18	0.35	2.26	0.59	1.34
0.12	6.58	0.36	2.19	0.60	1.32
0.13	6.08	0.37	2.13	0.61	1.30
0.14	5.64	0.38	2.08	0.62	1.27
0.15	5.27	0.39	2.03	0.63	1.25
0.16	4.94	0.40	1.98	0.64	1.23
0.17	4.65	0.41	1.93	0.65	1.22
0.18	4.39	0.42	1.88	0.66	1.20
0.19	4.16	0.43	1.84	0.67	1.18
0.20	3.95	0.44	1.80	0.68	1.16
0.21	3.76	0.45	1.76	0.69	1.14
0.22	3.59	0.46	1.72	0.70	1.13
0.23	3.43	0.47	1.68	0.71	1.11
0.24	3.29	0.48	1.65	0.72	1.10
0.25	3.16	0.49	1.61	0.73	1.08
0.26	3.04	0.50	1.58	0.74	1.07
0.27	2.93	0.51	1.55	0.75	1.05
0.28	2.82	0.52	1.52	0.76	1.04
0.29	2.72	0.53	1.49	0.77	1.03
0.30	2.63	0.54	1.46	0.78	1.01
0.31	2.55	0.55	1.44	0.79	1.00
0.32	2.47	0.56	1.41	0.80	0.99

* The factor 0.790 was determined from highly precise dilutions of a number of different plasma samples with a T-1824 solution of the specified optical characteristics. Since these measurements were in exact accord, and since immediate duplications of this method on the same patient yield almost identical "V" values, it is believed that there is no significant variation in dye adsorption by different plasmas, that the adsorption by other tissues is not a factor within the stipulated time interval and that Table V-50A is generally applicable.

TABLE V-50B

Correction Factor "K"

$K = \frac{0.30}{D}$ when D is the *measured* optical density of the cuvette-"standard" dye combination

Optical Density D	Correction Factor K	Optical Density D	Correction Factor K	Optical Density D	Correction Factor K
0.280	1.071	0.305	0.984	0.325	0.923
0.285	1.053	0.310	0.968	0.330	0.909
0.290	1.034	0.315	0.952	0.335	0.896
0.295	1.017	0.320	0.938	0.340	0.882
0.300	1.000				

NOTES

ZINC TURBIDITY

A spectrophotometric adaptation based on the methods and modifications of Kunkel and Hoagland (1947) and Kunkel (1947)

PRINCIPLE

Under certain conditions, a turbidity is formed when serum is treated with a buffered zinc solution which is apparently related to the concentration of serum gamma globulin. The turbidity is measured nephelometrically, the density being obtained directly from the calibration curve for thymol turbidity (Method T-20) and expressed in terms of zinc units.

DETERMINATION IN SERUM

SAMPLE

Serum from blood withdrawn during post-absorptive state. Although precautions should be taken to avoid hemolysis, liberated hemoglobin, unless excessive, shows negligible absorption at the wave length used. The same wave length also largely eliminates the interference caused by bilirubin.

TEST PROCEDURE

Special Apparatus

Micro pipettes, accurately calibrated "to contain" 0.1 ml.

Volumetric flasks and pipettes to be within the tolerances specified by the National Bureau of Standards.

Reagent

1. **Zinc-Barbital Buffer Solution, pH 7.5 (Kunkel).** In a 1000 ml Florence flask place 280 mg barbituric acid ($C_4H_4O_3N_2 \cdot 2H_2O$—Merck, U.S.P.) and 210 mg sodium diethyl barbiturate ($C_8H_{11}O_3N_2Na$—Merck, U.S.P.); add approximately 900 ml distilled water and shake until dissolved, using gentle heat if necessary; allow to cool and add exactly 24 mg crystalline zinc sulfate ($ZnSO_4 \cdot 7H_2O$—Baker & Adamson, A.C.S.); shake until dissolved; transfer to a 1000 ml volumetric flask and dilute to volume with distilled water. Check pH potentiometrically and, if necessary, adjust by the addition of either finely powdered barbituric acid or sodium diethyl barbiturate as required and checking after each addition.

Technic

Note: Sera which have been preserved in the refrigerator must be allowed to come to a temperature of 21–25° C. before adding the buffered zinc.

1. In a cuvette (sample) place
   6 ml zinc-barbital buffer (1)
   0.1 ml serum from a micro pipette, thoroughly rinsing out the pipette by drawing in and expelling some of the mixture several times.
2. In another cuvette (reagent blank) place
   6 ml zinc-barbital buffer (1)
   0.1 ml distilled water.
3. Allow to stand at 25° C. for 30 minutes.
4. Mix thoroughly by inversion.
5. Measure the transmittancy of the sample against the reagent blank set at 100% transmission at a wave length of 650 mμ and obtain the zinc unit equivalent directly from the calibration table for thymol turbidity (Method T-20).

CALIBRATION PROCEDURE

This calibration procedure need not be carried out. The values are obtained directly from the calibration table for thymol turbidity (Method T-20).

PROCEDURE FOR USE WITH LINEAR-SCALE PHOTOELECTRIC COLORIMETERS (SHEARD-SANFORD TYPE)

TEST PROCEDURE

Exactly as outlined; set the reagent blank at 100 and use a red filter.

CALIBRATION PROCEDURE

Need not be performed—values obtained directly from the calibration table for thymol turbidity.

PROCEDURE FOR USE WITH LOGARITHMIC-SCALE PHOTOELECTRIC COLORIMETERS (KLETT-SUMMERSON TYPE)

TEST PROCEDURE

Exactly as outlined; set the reagent blank at 0 and use a red filter.

CALIBRATION PROCEDURE

Need not be performed—values obtained directly from the calibration table for thymol turbidity.

Note: For instruments with cells, cuvettes or tubes requiring a final volume of more than 6 ml, use double the amount of serum and zinc-barbital buffer in the test procedure. Values are obtained directly from the calibration table for thymol turbidity (Method T-20) without further correction.

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NOTES

APPENDIX

SHOWING DENSITY (L = 2-logG)
AGAINST GALVANOMETER READING (G)

		1 (.25)	2 (.50)	3 (.75)			1 (.25)	2 (.50)	3 (.75)
1	2.000	1.903	1.824	1.757	51	.2924	.2903	.2882	.2861
2	1.699	1.648	1.602	1.561	52	.2840	.2819	.2798	.2777
3	1.523	1.488	1.456	1.426	53	.2756	.2736	.2716	.2696
4	1.398	1.372	1.347	1.323	54	.2676	.2656	.2636	.2616
5	1.301	1.280	1.260	1.240	55	.2596	.2577	.2557	.2537
6	1.222	1.204	1.187	1.171	56	.2518	.2499	.2480	.2460
7	1.155	1.140	1.126	1.112	57	.2441	.2422	.2403	.2384
8	1.097	1.083	1.071	1.059	58	.2366	.2347	.2328	.2310
9	1.046	1.034	1.022	1.011	59	.2291	.2273	.2255	.2236
10	1.000	.989	.979	.969	60	.2218	.2200	.2182	.2164
11	.959	.949	.939	.930	61	.2147	.2129	.2111	.2093
12	.921	.912	.903	.894	62	.2076	.2059	.2041	.2024
13	.886	.878	.870	.862	63	.2007	.1990	.1973	.1956
14	.854	.846	.838	.831	64	.1939	.1922	.1905	.1888
15	.824	.817	.810	.803	65	.1871	.1855	.1838	.1821
16	.796	.789	.782	.776	66	.1805	.1788	.1772	.1756
17	.770	.763	.757	.751	67	.1739	.1723	.1707	.1691
18	.745	.739	.733	.727	68	.1675	.1659	.1643	.1627
19	.721	.716	.710	.704	69	.1612	.1596	.1580	.1565
20	.699	.694	.688	.683	70	.1549	.1534	.1518	.1503
21	.678	.673	.668	.663	71	.1487	.1472	.1457	.1442
22	.658	.653	.648	.643	72	.1427	.1412	.1397	.1382
23	.638	.634	.629	.624	73	.1367	.1352	.1337	.1322
24	.620	.615	.611	.606	74	.1308	.1293	.1278	.1264
25	.602	.598	.594	.589	75	.1249	.1235	.1221	.1206
26	.585	.581	.577	.573	76	.1192	.1177	.1163	.1149
27	.569	.565	.561	.557	77	.1135	.1121	.1107	.1093
28	.553	.549	.545	.542	78	.1079	.1065	.1051	.1037
29	.538	.534	.530	.527	79	.1024	.1010	.0996	.0982
30	.523	.520	.516	.512	80	.0969	.0955	.0942	.0928
31	.509	.505	.502	.498	81	.0915	.0901	.0888	.0875
32	.495	.491	.488	.485	82	.0862	.0848	.0835	.0822
33	.482	.478	.475	.472	83	.0809	.0796	.0783	.0770
34	.469	.465	.462	.459	84	.0757	.0744	.0731	.0718
35	.456	.453	.450	.447	85	.0706	.0693	.0680	.0667
36	.444	.441	.438	.435	86	.0655	.0642	.0630	.0617
37	.432	.429	.426	.423	87	.0605	.0593	.0580	.0568
38	.420	.417	.414	.412	88	.0555	.0543	.0531	.0518
39	.409	.406	.403	.401	89	.0505	.0494	.0482	.0470
40	.398	.395	.392	.390	90	.0458	.0446	.0434	.0422
41	.387	.385	.382	.380	91	.0410	.0398	.0386	.0374
42	.377	.374	.372	.369	92	.0362	.0351	.0339	.0327
43	.367	.364	.362	.359	93	.0315	.0304	.0292	.0281
44	.357	.354	.352	.349	94	.0269	.0257	.0246	.0235
45	.347	.344	.342	.340	95	.0223	.0212	.0200	.0188
46	.337	.335	.332	.330	96	.0177	.0166	.0155	.0144
47	.328	.325	.323	.321	97	.0132	.0121	.0110	.0099
48	.319	.317	.314	.312	98	.0088	.0077	.0066	.0055
49	.310	.308	.305	.303	99	.0044	.0033	.0022	.0011
50	.301	.299	.297	.295	100	.0000	.0000	.0000	.0000

CUVETTE DIAMETERS

The following remarks pertain only to Coleman spectrophotometers and cuvettes.

Coleman spectrophotometers are designed to permit the use of cuvettes of different diameters. The analytical data obtained with a cuvette of one diameter may be applied to a calibration curve or table constructed by means of a cuvette of another diameter provided that a factor to correct for the difference in diameters is used.

Square cuvettes in which the absorbing path of the solution is 13 mm were used in the preparation of the calibration curves in this manual. Within certain limitations, these curves may be used with cuvettes of other diameters by proceeding as follows:

> Obtain the apparent concentration from the original calibration curve or table and multiply by the diameter of the cuvettes used for the analysis divided by the diameter of the cuvette used for the calibration, or

$$\text{Apparent concentration} \times \frac{\text{Outside diameter of cuvette used for calibration}}{\text{Outside diameter of cuvette used for analysis}} = \text{True concentration}$$

The use of a different diameter is of definite advantage in extending or shortening the concentration range. For very pale solutions in which the transmittancy reading falls between 100 and 80%, a cuvette of larger diameter will shift the readings more to the center of the scale. The same effect is achieved with deeply colored solutions by using cuvettes of smaller diameters. Cuvettes of smaller diameter give flatter curves; those of larger diameter, steeper curves.

If the approximate concentration of the substance being determined is unknown and upon completion of the analysis it is found that the final color is too light or too dark for accurate reading, measurement with a cuvette of an appropriate diameter may often suffice and save the analysis from being repeated with a smaller or larger aliquot of sample. Occasionally only certain limited concentrations may be of interest and smaller or larger cuvettes may be used to shift the particular range to that part of the galvanometer scale affording the most accurate readings (between 35 and 40% transmission). Whenever manipulating cuvettes in such a manner, especially with calibration curves that are not strictly linear, care must be taken that the minimum transmittancy readings obtained with the new cuvette are not less than the minimum transmittancy reading of the calibration curve.

In this manual square cuvettes were used not only because the author prefers them, but because the 13 mm solution path is approximately in the middle of the range of available cuvette diameters and when necessary larger or smaller sizes may be used. It is better practice to vary the diameter of the cuvette rather than to increase or decrease the amount of sample beyond the limits stated in the respective methods or to increase or decrease the volume of final colored solution. It is still better practice to repeat the calibration procedure and plot a new curve with the preferred cuvette size.

Note: Cuvettes, even though of the same diameter, may be of a different series, and are so marked by the manufacturer. For comparable results cuvettes with which the analytical data are obtained and those used for the preparation of the calibration curve must be of the same series.

PREPARATION OF MISCELLANEOUS SOLUTIONS

Sodium Hydroxide, Concentrated, Carbonate-Free. In a 6 liter Florence flask place 4000 ml distilled water, add slowly, while constantly shaking the flask under cold running tap water, 4000 gm sodium hydroxide pellets (NaOH—Baker & Adamson, A.C.S.); stir until solution is complete. While the solution is still hot, insert into the neck of the flask a 2-hole rubber stopper fitted with a soda-lime guard and a syphon tube, one end of which reaches nearly to the bottom of the flask; a rubber tube with a pinchcock is fitted to the outlet end. Allow to stand until sedimentation is complete and the supernatant fluid is clear—this may take a week or longer. The supernatant fluid is syphoned off as needed without disturbing the sediment.

The approximate strength of this solution is determined as follows:

> Accurately pipette 1 ml of the concentrated hydroxide solution into a 200 ml volumetric flask, dilute to volume with distilled water and mix thoroughly; transfer 25 ml to a porcelain evaporating dish, add 2 drops 1% phenolphthalein and titrate against standard 0.1 N acid—the ml of 0.1 N acid required × 3.2 = gm sodium hydroxide per 100 ml concentrated solution.

When a definite weight of sodium hydroxide is required the volume of concentrated solution containing the required amount is determined as follows:

$$\frac{\text{Amount required in gm}}{\text{Concentration of stock solution in gm per 100 ml}} \times 100 = \text{Ml stock solution containing the required amount}$$

Dichromate-Sulfuric Acid Cleaning Solution. In a 2 liter Florence flask place 20 gm technical grade sodium dichromate; dissolve in the minimum amount of water; with constant shaking and while cooling under running tap water, slowly add 1000 ml concentrated technical grade sulfuric acid; cover the flask and allow to cool. Replace when the color changes from deep orange-brown to greenish.

TABLE OF NORMAL VALUES

Albumin
  Plasma or serum—4.5 (3.8–6.7) gm per 100 ml

A/G Ratio—1.4–2.0

Amino Acid Nitrogen, Total
  Whole blood—6 (5–8) mg per 100 ml
  Plasma—4–5 mg per 100 ml
  Serum—5–6 mg per 100 ml
  Urine—200–700 mg per day

Ammonia Nitrogen
  Whole blood—0.1–0.2 mg per 100 ml
  Urine—0.7(0.5–1.0) gm per day

Amylase
  Plasma or serum—b-Amylose cotton (Method A-35)—75 (40–145) units
    Iodine-starch (Method A-36)—70 (45–135) units
    Starch substrate (Method A-37)—80–150 units

Ascorbic Acid
  Whole blood, plasma or serum—0.6–2.5 mg per 100 ml
  Urine—20 (15–25) mg per day

Bilirubin
  Plasma or serum—0.2–0.8 mg per 100 ml

Calcium
  Plasma or serum—
    Adults—9–11.5 mg per 100 ml; 4.5–5.6 meq per liter
    Children—10–11.5 mg per 100 ml; 5–5.6 meq per liter
    Infants—10.5–12 mg per 100 ml; 5.2–6 meq per liter
  Urine—50–300 mg per day
  Feces—400–800 mg per day

Carbon Dioxide Capacity
  Plasma—Adults—50–80 volumes per cent
    Infants—40–55 volumes per cent

Carbon Dioxide Content
  Plasma—Arterial blood—45–55 volumes per cent
    Venous blood—50–60 volumes per cent

Chloride
  Plasma or serum—365 (355–376) mg Cl per 100 ml;
    103 (100–106) meq per liter
    600 (570–630) mg NaCl per 100 ml
  Urine—10–15 gm NaCl per day
  Cerebrospinal fluid—720–760 mg NaCl per 100 ml
  Gastric contents—550 mg Cl per 100 ml

Cholesterol, Total
  Plasma or serum—Male—150–240 mg per 100 ml
    Female—180–260 mg per 100 ml

Cholesterol, Free—20–40% of total cholesterol

Cholesterol, Ester—60–80% of total cholesterol

Copper
  Whole blood—0.13 (0.05–0.25) mg per 100 ml
  Plasma or serum—Male—0.116 (0.082–0.134) mg per 100 ml
    Female—0.131 (0.103–0.159) mg per 100 ml

Creatine
  Whole blood—3–7 mg per 100 ml
  Plasma or serum—0.4–0.8 mg per 100 ml
  Urine—Male—0–50 mg per day
    Female—0–200 mg per day
    Children—10–50 mg per day

Creatinine
  Whole blood, plasma or serum—1 (0.8–1.7) mg per 100 ml
  Urine—1.25 (0.7–2.0) gm per day

"Dextrose" (Total Reducing Substances)
  Whole Blood—80–120 mg per 100 ml
  Plasma or serum—70–100 mg per 100 ml

Note: Normal values for arterial and venous samples are comparable only if the blood is withdrawn during the post-absorptive state.

  Urine—50–200 mg per day
  Cerebrospinal fluid—40–80 mg per 100 ml

Dextrose, True
  Whole blood, plasma or serum—
    Macro—70–90 mg per 100 ml
    Micro—73–85 mg per 100 ml

Ethyl Alcohol
  Whole blood—0–4 mg per 100 ml

Fibrinogen
  Plasma—300 (200–600) mg per 100 ml

Globulin
  Plasma or serum—2.5 (1.2–3.2) gm per 100 ml

Guanidine
  Whole blood—0.2–0.3 mg per 100 ml
  Urine—3–10 mg per day

Hemoglobin
  Whole blood—Male—15.6(14–18) gm per 100 ml
    Female—14.3 (12–16) gm per 100 ml
    Children—10–26 gm per 100 ml

Note: Normal values are dependent upon age, sex, locality and a number of other factors.

Hydrogen Ion Concentration
  Plasma or serum—pH 7.3–7.5

Note: Normal values are about 0.1 pH unit lower in arterial than in venous blood.

  Urine—pH 6.0 (4.8–8.0)

Indican
  Plasma or serum—0.05–0.15 mg per 100 ml
  Urine—40–150 mg per day

Iodine, Total
  Whole blood—12 (6–15) gamma per 100 ml
  Urine—50 (10–200) gamma per day

Iodine, Protein-Bound
  Whole blood—2–4 gamma per 100 ml
  Plasma or serum—4–8 gamma per 100 ml

Iodine, Inorganic
  Plasma or serum—2 (0.5–4.0) gamma per 100 ml

Iron, Non-Hemoglobin
  Plasma or serum—0.1 (0.05–0.25) mg per 100 ml

- Iron, Total
  - Whole blood—Male—52 (47–57) mg per 100 ml
    - Female—48 (43–53) mg per 100 ml
- 17-Ketosteroids, Total
  - Urine—Male—15 (10–22) mg per day
    - Female—12 (6–18) mg per day

  Note: Normal values without separation of the non-ketonic fraction are 10–25% higher.
- Lactic Acid
  - Whole blood—5–20 mg per 100 ml
  - Urine—50–200 mg per day
- Lead
  - Urine—10–100 gamma per liter
- Magnesium
  - Whole blood—1.6 mg per 100 ml; 1.3 meq per liter
  - Plasma or serum—2.5 (1–3) mg per 100 ml; 2.06 (0.82–2.5) meq per liter
  - Urine—0.05–0.2 gm per day
- Nicotinic Acid
  - Red blood cells—13 (12–14) gamma per ml
- Nicotinic Acid Derivatives
  - Urine—Exclusive of trigonelline—1–3 mg per day
    - Trigonelline (coffee-free diet)—20–29 mg per day
    - Trigonelline (coffee in diet)—to 200 mg per day
- Nonprotein Nitrogen
  - Whole blood—25–35 mg per 100 ml
  - Plasma or serum—20–30 mg per 100 ml
- Nitrogen, Total
  - Whole blood—3–3.7%
  - Plasma or serum—1.2 (0.9–1.4)%
  - Urine—12–18 gm per day
- Oxygen Capacity
  - Whole blood—Male—20.7 (16–24) volumes per cent
    - Female—19 (16–24) volumes per cent
- Oxygen Content
  - Whole blood—Arterial blood—15–23 volumes per cent
    - Venous blood—10–18 volumes per cent
- Phenol, Free
  - Whole blood—0.0184–0.063 mg per 100 ml
  - Urine—0.3 (0.2–0.4) mg per day
- Phenol, Total
  - Whole blood—0.055–0.12 mg per 100 ml
  - Urine—31 (15–45) mg per day
- Phenol, Conjugated
  - Whole blood—0.04–0.0695 mg per 100 ml
  - Urine—31 (16–45) mg per day
- Phosphatase, Acid
  - Serum—Bodansky—0–0.64 unit
    - Gutman—0–3.5 units
    - King-Armstrong—3 (0–5) units
    - Shinowara-Jones-Reinhart—0–1.1 units
- Phosphatase, Alkaline
  - Serum—Bodansky—Adults—2.7 (1.5–4.0) units
    - Children—8 (5–15) units
    - King-Armstrong—0–13 units
    - Shinowara-Jones-Reinhart—2–9 units
- Phosphorus, Total Acid-Soluble
  - Whole blood—26 (20–31) mg per 100 ml
  - Plasma or serum—2–6 mg per 100 ml
- Phosphorus, Inorganic
  - Whole blood—3.8 (3.0–4.7) mg per 100 ml
  - Plasma or serum—Adults—3.7 (2.5–4.5) mg per 100 ml
    - Children—4–6 mg per 100 ml
    - Infants—5.5–6.5 mg per 100 ml
  - Urine—1–1.5 gm per day
- Phosphorus, Lipoid
  - Whole blood—14 (10–18) mg per 100 ml
  - Plasma or serum—7–14 mg per 100 ml
- Phosphorus, Total
  - Whole blood—40 (35–45) mg per 100 ml
  - Plasma or serum—16–20 mg per 100 ml
  - Urine–1.1 gm per day
- Potassium
  - Serum—18.5 (16–22) mg per 100 ml; 4.1–5.6 meq per liter
- Pregnandiol
  - Urine—Male—0.2 mg per day
    - Female, non-pregnant—0.5–5.0 mg per day
      - pregnant—10–125 mg per day, depending upon the stage of the pregnancy
- Protein, Total
  - Plasma or serum—7 (6–8.2) gm per 100 ml
  - Cerebrospinal fluid—15–45 mg per 100 ml
  - Urine—less than 75 mg per day
- Pyruvic Acid
  - Whole blood—0.6–1.2 mg per 100 ml
  - Urine—2 mg per 100 ml
- Sodium
  - Serum—330 (315–345) mg per 100 ml; 143 (138–150) meq per liter
- Sulfate, Inorganic
  - Whole blood—0.4–1.1 mg S per 100 ml
  - Plasma or serum—1 (0.9–1.1) mg S per 100 ml
  - Urine—90% of total sulfate
- Sulfate, Ethereal
  - Whole blood—0.9–1.5 mg S per 100 ml
  - Plasma or serum—0.6–1.0 mg S per 100 ml
  - Urine—0.04–0.1 gm S per day
- Sulfate, Total
  - Whole blood—1.9–2.0 mg S per 100 ml
  - Plasma or serum—1–1.5 mg S per 100 ml
  - Urine—0.8 (0.6–1.2) gm S per day
- Sulfur, Total
  - Whole blood—3.8–4.5 mg S per 100 ml
  - Urine—1 gm S per day
- Sulfur, Neutral
  - Whole blood—2.2–2.5 mg S per 100 ml
  - Urine—0.08–0.16 gm S per day
- Thymol Turbidity
  - Serum—0–5 units

Urea Nitrogen
  Whole blood—8–15 mg per 100 ml
  Plasma or serum—10–17 mg per 100 ml
  Urine—7–15 gm per day; 10–40 gm urea

Note: Normal values are dependent upon the amount of protein in the diet.

Uric Acid
  Whole blood—3 (2–4.6) mg per 100 ml
  Plasma or serum—3–5 mg per 100 ml
  Urine—0.7 (0.4–1.0) gm per day

Urobilinogen
  Urine—0.2–3.0 mg per day
  Feces—30–200 mg per 100 gm

Volume
  Total blood—Male—70–85 ml per kg
    Female—59–73 ml per kg
  Total plasma—Male—40–48 ml per kg
    Female—37–46 ml per kg

Zinc Turbidity
  Serum—8 (2–19) units

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